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HEMOPOIETIC COLONIES ON THE CHORIOALLANTOIC MEMBRANE OF THE CHICK EMBRYO: INDUCTION BY EMBRYONIC, ADHERENT, NON HEMOPOIETIC SPLEEN CELLS

by

© GORDON M. KELLER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled HEMOPOIETIC COLONIES ON THE CHORIOALLANTOIC MEMBRANE OF THE CHICK EMBRYO: INDUCTION BY EMBRYONIC, ADHERENT, NON HEMOPOIETIC SPLEEN CELLS submitted by GORDON M. KELLER in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in IMMUNOLOGY.

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Abstract

Granulocytic and erythrocytic colonies developed on the chick embryo chorioallantoic membrane (CAM) following the inoculation of chick embryo spleen cells. Dose response and kinetic experiments showed that the colonies are derived from cell aggregates present in the inoculum. Dissociation and reaggregation kinetics of the CAM colony-forming cells (CAM-CFC) indicate that the cells must be present as aggregates in order to form colonies. Results from the morphological and cell marker studies suggest that the colony forming aggregates (CAM-CFA) attract and support the differentiation of primitive host hemopoietic cells. The physical characteristics of the CAM-CFC, which are different from those of the hemopoietic progenitor cells, indicate that they represent a stromal cell population of the chick embryo spleen. Further evidence supporting this notion was provided by the radiation studies which showed that the colony-forming ability of the CAM-CFC is relatively radioresistant. The above characteristics of the CAM-CFC suggest that they represent the hemopoietic microenvironment of the chick embryo spleen.

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Abbreviations

CFU-S:	Colony forming units in spleen
CFU-C:	Colony forming units in culture
GM-CFC:	Granulocyte-macrophage colony forming cell
CFU-E:	Erythroid colony forming unit
BFU-E:	Erythroid burst forming unit
EO-CFC:	Eosinophil colony forming cell
MEG-CFC:	Megakaryocyte colony forming cell
BL-CFC:	B-lymphocyte colony forming cell
EPO:	Erythropoietin
GM-CSF:	Granulocyte-macrophage colony stimulating factor
CSA:	Colony stimulating activity
MGI:	Macrophage-granulocyte inducer
MEG-CSF:	Megakaryocyte colony stimulating factor
EO-CSF:	Eosinophil colony stimulating factor
BPA:	Burst promoting activity
BFA:	Burst forming activity
HLCM:	Human leukocyte conditioned medium
PGE:	Prostaglandin E
TSRC:	Theta-sensitive regulatory cell
HIM:	Hemopoietic Inductive Microenvironment
CAM:	Chorioallantoic membrane
CAM-CFA:	Chorioallantoic membrane colony forming aggregate
CAM-CFC:	Chorioallantoic membrane colony forming cell

Chapter I

Literature Review

A. Mature Blood Cells - Morphology and Function

Mature blood cells* are usually divided into three broad classes: (1) erythrocytes, (2) leukocytes and (3) thrombocytes (in birds) or megakaryocytes (in mammals) (Lucas and Jamroz, '61; Metcalf and Moore, '71). The leukocytes can be further subdivided into lymphocytes, granulocytes and monocytes.

Mammalian erythrocytes (or red blood cells) are small, biconcave cells which have differentiated to an end cell stage where they no longer contain a nucleus. Avian red cells, on the other hand, are large oblong shaped, nucleated cells. The function of the erythrocytes is well established and involves esentially oxygen transport.

The group of cells commonly known as the granulocytes consists of 3 different cells types: neutrophils (mammals) or heterophils (birds), eosinophils and basophils. (Reviewed in Lucas and Jamroz, '61; Archer, '70; Metcalf and Moore, '71; Cline, '75; Linman, '75; Parwaresch, '76; Williams et al., '77.)

Mammalian neutrophils are medium sized cells with

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*This review will deal primarily with studies on mammalian and avian blood cells, however, in a few specific cases those from other vertebrate classes will be included.

numerous, fine, evenly distributed, blue-violet cytoplasmic granules (under Wright's stain). The avian counterpart of the neutrophil, the heterophil, contains cytoplasmic rods, granules, or non-staining vacuoles. The rods, which vary considerably in length and thickness, as well as the granules stain with eosin dye. The nucleus of the neutrophils and heterophils is usually lobulated, in some instances consisting of 5 individual lobes that vary in size and shape. Through their ability to phagocytose foreign material, these cells constitute one of the first lines of defense in the body.

The cytoplasm of eosinophils contains refractile, eosinophilic (red-orange staining) granules, which, in the mammals, are considerably larger than those found in the neutrophils. Although both avian eosinophils and heterophils contain eosinophilic granules, these cells can be differentiated on the basis of nuclear and background cytoplasmic staining. The nucleus of eosinophils is usually bilobed, although infrequently cells with multilobed nuclei are found. Eosinophils are able to phagocytose foreign material, however, in these cells the process appears to be less efficient than in neutrophils. Eosinophils are present in high numbers in helminth infections, allergic states and almost any inflamatory condition. Their appearance in allergic reactions and inflamatory situations may be related to their apparent ability to detoxify some of the chemical mediators involved in these reactions. Recent studies

suggest that the eosinophilia associated with helminth infections reflects the ability of these cells to destroy parasites (Butterworth et al., '77).

Basophils, as the name indicates, contain large basophilic (deep purple staining) granules which often tend to overlie the single- or bi-lobed nucleus. Blood basophils appear to be closely related to tissue mast cells, both of which play a role in the pathogenesis of immediate hypersensitivity reactions and allergic states. In addition, blood basophils appear to be capable of clearing fat from the blood stream.

Lymphocytes are the only class of differentiated blood cells capable of further division (Roitt, '74; Linman, '75; Cline, '75; Williams et al., '77). Several different forms are found in the blood. Large lymphocytes have abundant cytoplasm which may contain a few small, sharply defined, azurophilic granules. The dense staining nucleus may be round or slightly indented. Small lymphocytes are similar in appearance, although they contain considerably less cytoplasm. Lymphocytes are primarily involved in combating the invasion of foreign organisms. Depending upon the nature of the foreign material, the immune response manifested by lymphocytes may be humoral or cellular. Recent data indicates that some classes of lymphocytes are involved in the regulation of hemopoietic differentiation as well (Goodman and Shinpock, '68; Wiktor-Jedrzejczak et al., '77;

Metcalf, '77).

Blood monocytes are similar in size to the large lymphocytes and have a grey-blue staining cytoplasm which may contain some azurophilic granules as well as a few small vacuoles (Cline, '75; Linman, '75; van Furth, '75; Williams et al., '77). The nucleus is usually round or kidney-shaped, although, in some instances it may be lobulated. There is now a considerable amount of evidence suggesting that blood monocytes are precursors of the tissue macrophages. The function of the monocyte-macrophage line of cells involves phagocytosis of invading microorganisms and devitalized cells, processing antigens for presentation to lymphoid cells, defense against tumors, and regulation of hemopoiesis.

Blood platelets in mammals appear as small fragments of non-nucleated cytoplasm which have become detached from the periphery of the megakaryocytes (Linman, '75; Williams et al., '77). The avian equivalent of the platelet, the thrombocyte, is a relatively small nucleated cell derived from an antecedent mononucleated blast cell (Lucas and Jamroz, '61). Thrombocytes (and platelets) have a critical role in response to injury that involves thrombus formation, hemostasis and vascular and connective tissue healing.

B. Historical Aspects of Hemopoiesis

At the beginning of this century the major controversy

in hematology was centered around the origin of the different classes of blood cells.

Two opposing groups of theories were put forward describing the differentiation of stem cells into mature progeny (Reviewed by Bloom, '37). The polyphyletic theories proposed that two or more stem cells exist, each restricted to one or two lines of differentiation while the monophyletic theories stated that all blood cells originate from a single multipotent stem cell. Of the first category, the most widely accepted was the trialistic theory described by Schilling and the dualistic theory, initially introduced by Erhlich and Lazarus and later modified by Schriddle. The basic difference between the two is that the trialists encorporated three different stem cells into their model of hemopoiesis (separate stem cell for lymphocytes, monocytes, and remainder of hemopoietic cells), whereas the dualist scheme contained only two (one for the lymphocytes and one for the myeloid cells). One of the most popular monophyletic theories was proposed by Maximow. He believed all blood cells originated from a common stem cell that he called the hemocytoblast. Furthermore, he suggested that differences in organ environment as well as other factors were responsible for determining the differentiation pathway of a particular stem cell.

The major fault of both groups of theories is that they were based solely on histological studies and acceptance of

either depended upon one's interpretation of static morphological sequences. For this reason neither theory could be unequivocally proved or disproved and thus the controversy remained until techniques were introduced that allowed the progeny of a single cell to be positively identified.

Although it was not realized at the time, the pioneering studies of Jacobson et al. ('49) provided the first experimental evidence demonstrating the existence of hemopoietic stem cells. They found that radiation death in mice could be greatly reduced by shielding the spleen during the irradiation procedure. They went on to show that isologous and autologous young spleens grafted into irradiated animals have similar therapeutic effects (Jacobson et al., '51). Since very little was known about the migratory ability of hemopoietic cells, it was thought that the spleens provide a radio sensitive factor, capable of stimulating the regeneration of blood forming tissue. In the same year, Lorenz et al. ('51) demonstrated that lethally irradiated mice and guinea pigs can be protected by intravenous or intraperitoneal injection of isologous bone marrow cells. In subsequent studies they showed that both homologous and heterologous bone marrow cells are effective in protecting against radiation death (Lorenz et al., '52; Lorenz and Congdon, '54). They argued that these results further support the notion that cells provide a protective humoral factor since it was inconceivable that heterologous

cells could repopulate an irradiated host and provide cellular function.

This "humoral hypothesis" was soon dismissed by Ford and his colleagues ('56, '57), who repopulated irradiated mice with either rat cells or chromosomally marked (T6) mouse cells, both of which can be distinguished from the normal host cells. Shortly after irradiation and injection of such cells, it was found that a large majority of the cell population in the various hemopoietic organs is of donor origin. The mice repopulated with cells containing the T6 marked chromosome appear to be stable chimeras, since donor cells predominate in the hemopoietic tissue for more than 300 days after irradiation and injection. These findings strongly suggest that the graft provides functional cells rather than a humoral factor.

In contrast, a number of the mice repopulated with rat cells are less stable and in some cases host cells begin to appear in high numbers several months after irradiation (Ford et al., '57; Barnes et al., '59). Some of the host cells which retain their proliferative capacity contain radiation induced chromosome alterations. Since the chromosome damage is random, each cell is unique and thus cells with identical abnormal karyotypes belong to the same clones. With this in mind, Barnes et al. ('59) carefully analyzed a number of mice in which the hemopoietic system had reverted back to host cells. In one particular mouse,

95% of the bone marrow, 55% of the spleen, 80% of the thymus and 67% of the lymph mode cells contained an identical unique karyotype, strongly suggesting that all these cells are the progeny of a single precursor cell. That a single clone apparently repopulated both the hemopoietic and lymphoid organs, was the first indication that pluripotent stem cells exist.

C. The Pluripotent Stem Cell

1) The CFU Assay - An Assay for Pluripotent Stem Cells

It was not until Till and McCulloch introduced the CFU assay that the existance of the pluripotent stem cell was unequivocally demonstrated.

Nearly two decades ago, these investigators found that macroscopically visible nodules develop in the spleen of irradiated mice following the injection of small numbers of isologous bone marrow cells (Till and McCulloch, '61). These nodules or colonies are foci of hemopoiesis that contain differentiating erythroid, granulocytic, eosinophilic or megakaryocytic cells. Early in development (6-10 days) such colonies consist of only a single line of differentiation (Lewis and Trobaugh, '64; Bleiberg et al., '67; Curry and Trentin, '67; Metcalf and Moore, '71). As they increase in age, (12-14 days) most colonies become mixed, some containing as many as three different cell types.

A single cell origin of the colonies is indicated by

the linearity and absence of an initial threshold in the cell dose response curve. Furthermore, radiation survival curves, indicating that colony formation is radio sensitive, resemble curves obtained for single cells in culture (Puck and Marcus, '56).

Direct proof for the clonality of the colonies was provided by Becker et al. ('63) who generated spleen colonies with abnormal karyotypes by irradiating donor cells prior to injection. When colonies with unique karotypes were found, it was noted that a vast majority (>90%) of the cells contained this karyotype indicating that they are the progeny of a single cell. By incorporating differentiation markers (^{59}Fe uptake and peroxidase positivity) into the above experimental protocol, it was shown that, in mixed colonies, both erythrocytes and granulocytes belong to the same clone (Wu et al., '67). These findings clearly demonstrate that the macroscopic spleen colonies are derived from a single multipotent cell present in the bone marrow. The spleen colony forming cell has become known as the CFU-S (colony forming unit of the spleen) and since has been found in the peripheral blood, adult spleen, yolk sac, fetal spleen, and fetal liver (Micklem and Loutit, '66; Barnes and Loutit, '67; Silini et al., '67; Barker et al., '69; Worton et al., '69b; Moore and Metcalf, '70; Metcalf and Moore, '71).

Although lymphoid colonies are not found in the spleen,

there is convincing evidence that CFU-S can give rise to functional lymphocytes. Several groups have shown that a single colony or a small number of colonies can repopulate the lymphoid system of an irradiated mouse (Trentin and Fahlberg, '63; Trentin et al., '67; Mekori and Feldman, '65). This repopulation appears to be complete since such mice regain immunological competence with the ability to respond to a number of different antigens. Using a slightly different approach, Wu et al. ('68a) showed that pooled spleen colony cells, all carrying the same unique irradiation induced karyotype, are able to repopulate the lymph nodes of irradiated mice. Some of these lymph nodes were found to contain more than 65% marked cells. These studies together with others showing that the CFU-S can give rise to rosette forming cells (Edwards et al., '70) strongly suggest that the spleen colony forming cells are truly pluripotent, capable of giving rise to both the lymphoid and myeloid classes of mature blood cells.

More direct evidence for the existance of pluripotent cells comes from the recent studies of Johnson and Metcalf ('77) who showed that single fetal liver cells are able to generate mixed hemopoietic colonies in vitro.

In addition to the multipotency, the CFU-S' also possess the capacity to renew themselves. By dissecting out individual colonies and transplanting them into secondary irradiated recipients, Siminovitch et al. ('63) found that

most fourteen day old colonies contain CFU-S'. They noted, however, that the distribution of these cells is extremely heterogeneous with a large number of colonies containing a few CFU-S' and a small number of colonies containing many CFU-S'. Furthermore, they found no correlation between physical characteristics of the colonies and their CFU-S content.

Although, most colonies do contain CFU-S, the capacity of these cells to undergo extensive self-renewal does appear to be limited. Siminovitch et al. ('65) noted that CFU-S, transplanted repeatedly, lose their ability to generate spleen colonies and to protect from radiation death. Passaging the cells from pooled or individual colonies results in a quicker decline in the self-renewal capacity of the CFU-S'. Similar findings were reported by others who varied several of the experimental parameters such as graft size and time interval between cell passages (Cudkowicz et al., '64b; Lajtha and Schofield, '71; Pozzi et al., '73; Micklem and Ogden, '76).

Moore and Metcalf ('71) carried these studies one step further and demonstrated that marrow growth from old (2.5 years) mice and young (8 weeks) mice declines at the same rate. They did, however, find that CFU-S' from 9 day old yolk sac can be passaged for 6 to 7 generations and those from 15 day fetal liver have a passaging capacity intermediate between yolk sac and neonatal liver CFU-S which

can be transferred 4 times before they lose the ability to initiate spleen colonies.

The above studies strongly suggest that the CFU-S rapidly lose their self-renewal capacity following serial transplantation. One must, however, question the physiological relevance of such studies, since several investigators have found no difference in the functional abilities between old and young stem cells (Harrison, '73; Ogden and Micklem, '76; Harrison et al., '77). In fact, Harrison et al. ('78) have recently shown that young hemopoietic stem cells (from 3-8 month old mice) transplanted once are considerably less efficient in repopulating irradiated hosts than non passaged stem cells from old mice (25-33 months). These observations suggest that the loss of stem cell function reported in the previous experiments results from the stress associated with serial transplantation rather than from aging.

In summary, the CFU-S possess two of the characteristics common to all stem cells; (1) capacity for differentiation and (2) capacity for self-renewal. The spleen colony method, therefore, is a quantitative assay for hemopoietic stem cells.

2) Characteristics of the CFU-S

Physical characterization of the CFU-S from adult marrow has revealed an extremely heterogenous cell

population. Velocity sedimentation experiments show that the CFU-S sediment in the range of 3.0 - 5.0 mm/hour with a mode velocity of 3.9 mm/hour (Worton et al., '69b). Density centrifugation analysis reveal an even more heterogeneous population with peak numbers of CFU-S found at 1.063 gm/cc^3, 1.066 gm/cc^3 and 1.069 gm/cm^3 (Haskill et al., '70).

CFU-S from 10 day fetal liver, on the other hand, are considerably less dense and more homogenous (in density) than their adult counterparts (Moore et al., '70). Their density increases progressively throughout fetal development and reaches near adult values by eighteen days of gestation.

CFU-S' from normal adult marrow appear to be relatively quiescent. Using incorporation of high specific activity tritiated thymidine as a means of killing cells in the S phase, it was found that less than 10% of adult marrow CFU-S' are in cycle (Becker et al., '65; Lajtha et al., '69; Lahiri and Van Putten, '72). A slightly larger number of the CFU-S' from normal adult spleen was killed. Extensive killing was, however, observed with fetal liver (42% depression) and regenerating spleen (65% depression) CFU-S' indicating that a large number of these cells are cycling (Becker et al., '65).

The doubling time of the cycling CFU-S from adult regenerating tissue is between 20 and 25 hours (McCulloch and Till, '64; Schofield, '70; Metcalf and Moore, '71; Gidali et al., '74); a value which appears to remain

constant, regardless of the size of the regenerating graft. The CFU-S' from fetal liver have a slightly shorter doubling time of 16 hours (Schofield, '70).

Attempts at characterizing the hemopoietic stem cells morphologically have been relatively unsuccessful. Different groups described the CFU-S as a small or medium sized lymphocyte (Cudkowicz et al., '64a; Bennett and Cudkowicz, '68; Haas et al., '71), transitional cell (Yoffey, '69), and monocytoid type cell (Caffrey-Tyler and Everett, '66). More recent studies have described the CFU-S as a medium size cell with a large nucleus containing prominent nucleoli (Van Bekkum et al., '71; Dickie et al., '73; Van Bekkum, '76). In all these reports, however, there is no direct proof that the cells described as CFU-S can actually function as pluripotent stem cells.

Although morphological studies have been inconclusive, analysis of the surface markers on stem cells has provided valuable information. Golub ('72) discovered that mouse brain and the CFU-S share an antigen found on no other tissue. This brain associated antigen (BAS) is apparently species specific, since brain tissue from rats, chickens and humans could not absorb the activity from the sera. Recent studies indicate that the CFU-S also express both the K and D major histocompatibility antigens, however, they lack the Ia antigen as well as the Thy-1 antigen found on T lymphocytes (Van Den Engh et al., '78; Russell and Van Den

Engh, '79). Perhaps one of the more exciting findings is the presence of histamine H_2 receptors on the surface of the stem cell (Byron, '78). Although the studies are preliminary, there is some evidence suggesting that these receptors are involved in triggering the stem cell from Go into S phase of the cell cycle.

One important aspect of CFU-S requiring further investigation is that of heterogeneity. Cell separation experiments have shown the adult marrow CFU-S to be heterogeneous with respect to size and density (Worton et al., '69a; Haskill et a., '70). Further studies along this line indicate that these physical differences can be correlated with differences in self-renewal capacity (Worton et al., '69b), cell cycle (Monette et al., '74), and seeding efficiency (the ability to home to the spleen and form a colony) (Haskill et al., '70). More recently, a correlation between the CFU-S cell cycle and its position in the bone marrow has been found (Lord et al., '75). A large number of the CFU-S' near the bone appear to be cycling, while those in the more axial position of the marrow are in a quiescent state.

In addition to heterogeneity within the marrow CFU-S population, it has been shown that these stem cells differ from those found in the peripheral blood and spleen. Both the splenic and peripheral blood CFU-S appear to have a decreased self-renewal capacity compared to CFU-S in the

marrow (Micklem et al., '75; Lahiri and Van Putten, '69). It was also found that the peripheral blood and splenic CFU-S are deficient with respect to their ability to repopulate irradiated animals (Kretchmar and Conover, '70; Micklem et al., '75). These findings as well as those demonstrating differences in radio sensitivity (Lahiri and Van Putten, '72; Siminovitch et al., '65; Guzman and Lajtha, '70) and cycling activity (Becker et al., '65; Lahiri and Van Putten, '72; Guzman and Lajtha, '70) between splenic and marrow CFU-S' have led to the hypothesis that two classes of stem cells exist; (1) the primitive stem cells that are normally not cycling and have an extensive self-renewal capacity, and (2) the more differentiated cycling stem cells that have a limited self-renewal capacity (Micklem and Ogden, '76). Candidates for the former class would be the non-cycling CFU-S' found in the axial position of the marrow. The peripheral blood and splenic CFU-S' are thought to represent the latter class of stem cells. Progression of the CFU-S' from a primitive to more differentiated state would involve a change in cycling status, decreased capacity for self-renewal and perhaps a restriction in the differentiation potential. A recent study has indicated that stem cells restricted to myeloid differentiation do exist (Abramson et al., '77). Restriction of the differentiation potential might reflect a maturation process between the pluripotent stem cell and the committed progenitor cell.

3) Pluripotent Stem Cells in Other Species

All the experiments described thus far have involved mouse CFU-S, however, there is evidence for the presence of stem cells in rats and human as well.

As in mice, repopulation of irradiated rats with small numbers of rat bone marrow cells results in the formation of splenic hemopoietic colonies (Comas and Byrd, '67). A large majority (>95%) of these colonies appear to be erythrocytic, although, if rat bone marrow cells are injected into irradiated mice, splenic colonies (of rat cells) of all types develop in ratios similar to those found when the colonies are initiated by mouse CFU-S (Rauchwerger et al., '73b). The pluripotency of rat marrow stem cells was examined using a protocol similar to that of Wu et al. ('68a) in which chromosomal aberrations are induced in the donor cell population by irradiation. Some of the erythroid spleen colonies resulting from the injection of such cells into irradiated mice contained large numbers of cells with chromosomal abnormalities identical to those found in cells which responded in a mixed lymphocyte reaction (Nowell et al., '70). These findings strongly suggest that in rats, as in the mice, both the myeloid and lymphoid cells arise from a common ancestor cell.

Three lines of evidence suggest that pluripotent stem cells exist in humans.

First, the bone marrow cells from a large majority of patients suffering from chronic myelogenous leukemia (CML) contains a chromosomal aberration known as the Philadelphia Chromosome (Ph^1). This abnormality has been detected in erythroid, granulocytic and megakaryocytic marrow cells, suggesting that they are derived from a common stem cell (Whang et al., '63; Tough et al., '63).

Second, a small minority of female patients with CML or polycythemia vera are found to be heterozygous at the X-linked glucose-6-phosphate dehydrogenase (G-6-PD) locus. The result of inactivation of one of the X-chromosomes in XX somatic cells is that these females contain two populations of cells, one producing the type-B isoenzyme and the other type-A (Beutler et al., '62; Davidson et al., '63). In all cases observed thus far, the hemopoietic cells (granulocytes, erythrocytes, platelets, monocytes and more recently B- and sometimes T-lymphocytes) express only one form of the enzyme, suggesting that they belong to a single clone (Fialkow et al., '77, '78).

Finally, the most direct evidence for the existance of human pluripotent stem cells is provided by the recent studies of Fauser and Messner ('78, '79). They found that mixed hemopoietic colonies (some containing 4 different cell types) develop in cultures of human marrow and peripheral blood cells provided that spleen cell conditioned medium and erythropoietin are present. Y-chromatin analysis of colonies

derived from cell populations containing male and female cells, suggest that these mixed colonies are clones.

D. Committed Progenitor Cells

1) Granulocyte-Macrophage Progenitor Cells

a) Colony Formation in Vitro

Two separate studies showed that, when mouse bone marrow or spleen cells are cultured under suitable conditions in semisolid agar, colonies of cells develop (Pluznik and Sachs, '65; Bradley and Metcalf, '66). The colonies are composed of both neutrophilic granulocytes (referred to simply as granulocytes) and macrophages (Bradley and Metcalf, '66; Ichikawa et al., '66) and their development is entirely dependent upon the presence of a humoral inducer or factor known as granulocyte-macrophage colony stimulating factor (GM-CSF) (Paran and Sachs, '68; Metcalf and Foster, '67b; Metcalf, '70). A single cell origin of these colonies is suggested by the linearity of the curve relating the number of colonies formed to the number of cells plated (Pluznik and Sachs, '65; Bradley and Metcalf, '66). More recent studies by Moore et al. ('72), showing that single cells implanted into an agar culture form colonies containing both granulocytes and macrophages, clearly demonstrate that the colonies are clones. These cells, with an apparent dual differentiation potential, have become known as the colony forming units in culture (CFU-C) (Worton et al., '69b) or the granulocyte-macrophage colony

forming cell (GM-CFC) (Metcalf, '77). This review will deal primarily with adult and fetal mouse GM-CFCs, however, comparable cells have been found in a number of other mammals (Metcalf, '77) as well as in chickens (Dodge and Hansell, '78).

Although the morphology of both the colonies and the cells within the colonies varies depending upon the source of the GM-CFCs and the GM-CSF, colonies in a large majority of bone marrow cultures stimulated by high concentrations of GM-CSF follow the same basic pattern of development. Most begin as small clusters of differentiating granulocytes; they then become mixed containing both granulocytes and macrophages and finally they lose their granulocytic content and become purely macrophage colonies (Metcalf, '71b; Metcalf, '77). Differentiation is apparent as the cells within the young colonies are immature while those in the older colonies acquire characteristics of relatively mature granulocytes and macrophages (lobulated nuclei, granular cytoplasm, phagocytic activity) (Ichikawa et al., '66; Metcalf et al., '67b; Metcalf, '71b; Metcalf, '77).

A small number of the colonies do not pass through this transition phase and remain granulocytic. Deviations from the basic pattern of development are observed when colony forming cells from other sources are used. For example, cells from fetal liver (Moore and Williams, '73b; Johnson and Metcalf, '78a), the peritoneum of thioglycollate treated

mice (Lin and Stewart, '74) and the rapid sedimenting fractions after velocity sedimentation of adult bone marrow (Metcalf and MacDonald, '75) appear to initiate primarily macrophage colonies. The cells from the slower sedimenting fraction, on the other hand, give rise to more granulocytic colonies.

In addition to the source of GM-CFCs, both the concentration and the source of the GM-CSF can influence the type of cells within the colonies. At high concentrations of GM-CSF the development of granulocytic and mixed colonies is favoured, while at low concentrations more macrophage colonies are initiated (Metcalf and MacDonald, '75). Similarly, certain sources of GM-CSF will favour the growth of one type of colony over the other (Horiuchi and Ichikawa, '77; Johnson and Metcalf, '78b).

Along with large numbers of colonies, most agar cultures contain cell clusters (less than 50 cells) which are also composed of granulocytes and/or macrophages (Metcalf, '69; Metcalf and MacDonald, '75; Metcalf, '77). The cluster forming cells, which outnumber the GM-CFC by a ratio 5 to 1, appear to be more adherent (Metcalf et al., '71b) and slightly larger (Metcalf and MacDonald, '75) than the colony forming cells. In spite of these differences, the fact that cells from 3 and 7 day old colonies will initiate clusters upon transfer to secondary cultures (Metcalf and Foster, '67b), as well as the fact that GM-CFCs and cluster

forming cells share properties such as organ distribution (Metcalf, '77) and cell cycle status (Metcalf, '72) supports the interpretation that the cells which form clusters are the progeny of the colony forming cells.

b) Characteristics of the GM-CFC

Morphological studies have indicated that the GM-CFCs are approximately 8-10 microns in diameter, have a round or kidney-shaped nucleus, one or two prominent nucleoli, and a pale blue agranular cytoplasm (Moore et al., '72). The in vitro colony forming cells apparently do not express the major histocompatibility antigens or the brain associated stem cell antigen found on the surface of the CFU-S (Van Den Engh and Golub, '74). Cell separation experiments have shown that the GM-CFCs belong to a heterogenous cell population with densities ranging from 1.065 to 1.078 g/cc^3 and a mode sedimentation velocity of 4.3 mm/hr (Haskill et al., '70; Metcalf et al., '71b; Williams and Van Den Engh, '75; Metcalf and MacDonald '75). GM-CFCs from fetal liver are considerably less dense and sediment more quickly than those from adult tissues. Similar to the situation of the CFU-S, these characteristics of the GM-CFCs change during the latter stages of development, reaching near adult values shortly before birth (Moore et al., '70). Exposure to high concentration of tritrated thymidine or hydroxyurea leads to a 30-50% drop in the number of normal marrow GM-CFCs, while in a situation of active regeneration up to 80% of the GM-

CFCs are killed (Richard et al., '70; Iscove et al., '70; Metcalf, '72). As would be expected for a precursor cell, the GM-CFC were found to be highly radiosensitive, with an in vivo Do of 85-95 rads for mouse cells (Robinson et al., '67b; Chen and Schooley, '70) and an in vitro Do of 160 rads for human cells (Senn and McCulloch, '70).

c) Relationship Between the CFU-S and the GM-CFC

The experiments of Dickie et al. ('71) showing that CFU-S can be detected in agar colonies and those of Wu et al. ('68b) demonstrating a positive correlation between the number of CFU-S and GM-CFCs in individual spleen colonies suggests that the two cell types might be identical. There is, on the other hand, a considerable amount of evidence indicating that the CFU-S and GM-CFC belong to different cell populations.

A number of studies have shown that the GM-CFCs and the CFU-S differ with respect to their adherent properties (Metcalf et al., '71b), radiation sensitivity (Testa et al., '73), number in cycle (Lajtha et al., '69; Richard et al., '70), position in the bone marrow (Lord et al., '75) and antigens expressed on their surface (Van Den Engh and Golub, '74). In addition, velocity sedimentation experiments have indicated that the CFU-S sediment more slowly than most of the GM-CFCs (Worton et al., '69b), while density centrifugation analysis showed a clear separation between

the two populations in animals preinjected with Freund's adjuvant (Haskill et al., '70) or previously subjected to irradiation (Moore and Williams, '73a). Finally, in W/W^{v} mutant mice with genetically determined anemia, the number of CFU-S is extremely low while the number of GM-CFCs is close to normal (Bennett et al., '68). These observations strongly suggest that the CFU-S and the GM-CFC represent different cell population.

Other findings indicate that although the two cell types are different, they are closely related and most likely represent a parent-progeny relationship. Following fractionation of adult marrow cells by density centrifugation, Haskill et al. ('70) found that fractions containing CFU-S but devoid of GM-CFCs are able to repopulate irradiated mice and regenerate a full complement of hemopoietic cells, including large numbers of in vitro colony forming cells. In other studies, fractionated mouse marrow populations were cultured over kidney tubules and then tested for their GM-CFC content (Sutherland et al., '71). It was found that the slow sedimenting fractions containing the largest number of CFU-S generate the most GM-CFCs. Finally, using radiation induced cell markers, Wu et al. ('68b) discovered that clones of cells derived from CFU-S contain GM-CFCs.

These observations, together with those suggesting that the GM-CFCs are restricted in their differentiation

potential (Metcalf, '77; Metcalf and Johnson, '79) and have little or no capacity for self-renewal (Metcalf, '77) strongly support the notion that the in vitro colony forming cells are slightly more differentiated than CFU-S and most likely represent their immediate progency. Though the GM-CFC is not restricted to a single line of hemopoiesis, it is referred to as the progenitor of the granulocyte and macrophage lines of hemopoiesis.

2) Erythroid Progenitor Cells

a) Erythroid Colony Formation in Vitro

Stephenson et al. ('71) were the first to report the development of erythroid colonies in plasma gel cultures of fetal liver cells. Growth of these colonies is dependent on the presence of erythropoietin (EPO), a known humoral regulator of erythropoiesis (Krantz and Jacobson, '70). These erythroid colonies appear within 2 days of plating and reach maximum size (up to 60 cells) and numbers between 3 and 4 days of incubation. A differentiation process is apparent within the cultures as a majority of the early colonies contain basophilic erythroblasts while those at later stages consist primarily of normoblasts.

Improvement of the culture system by McLeod et al. ('74), permitting the growth of erythroid colonies from adult bone marrow cells, resulted in a more complete differentiation process with extrusion of the nuclei from

some of the red cells. The bone marrow colonies are somewhat smaller than those derived from fetal liver cells and reach peak numbers within 48 hours of plating. Colony formation at low cell numbers conforms to a poisson distribution suggesting that each colony is initiated by a single erythropoietin sensitive colony forming unit (CFU-E). Recently Cormak ('76), using time lapse photography on a monolayer of fetal liver cells, conclusively demonstrated that the CFU-E is a single cell.

If the plasma cultures are restimulated with EPO or if methyl cellulose cultures are initiated with a single high EPO dose, large burst colonies are found after 7 to 10 days of incubation (Axelrad et al., '73; Iscove and Sieber, '75). These bursts can contain up to 10^4 cells and are composed of a loose collection of aggregates each of which corresponds in size and cellular composition to the 48 hour colonies. Dose response studies indicate that each burst arises from a single burst forming unit (BFU-E). More recent experiments using a G-banding technique on colonies initiated by suspensions containing male and female cells strongly suggest that the BFU-E is a single cell (Strome et al., '78).

In addition to these late developing bursts, it has been found that a second type of burst develops at 3 days of incubation (Gregory, '76); the time at which the number of 48 hour colonies begins to decrease. These so called 3 day

bursts reach a maximum size of 150 cells by 4 days and then begin to decline.

b) Relationship Between BFU-E and CFU-E

Most studies indicate that the BFU-E and the CFU-E represent different, although related, cell populations.

The CFU-E appear to be rapidly sedimenting (Heath et al., '76; Wagemaker et al., '76), cycling cells (Iscove, '77; Hara and Ogawa, '77) with a limited proliferative potential (Axelrad et al., '78). The population size of the CFU-E fluctuates rapidly in response to treatments affecting the EPO level (anemia, plethora) (Gregory et al., '74; Wagemaker et al., '76; Iscover, '77), and in vitro, the CFU-E respond to relatively low concentrations of EPO (Iscove and Sieber, '75; Gregory, '76).

BFU-E, on the other hand, belong to a slow sedimenting cell population of which only 35% are cycling (Heath et al., '76; Wagemaker et al., '76; Hara and Ogawa, '77; Iscove, '77). The proliferative potential of these cells appears to be quite extensive as bursts of up to 10^4 cells can develop within 10 days. Hypertransfusion and bleeding have only minimal effects on the BFU-E population (Wagemaker, '76; Iscove, '77) and the changes that do occur in the different organs in vivo appear to be the result of migration rather than proliferation (Hara and Ogawa, '77). In vitro, the BFU-E normally respond only to high concentrations of EPO

(Iscover and Sieber, '75; Gregory, '76), however, recent studies indicate that the amount of EPO required for burst formation is significantly reduced if a burst promoting factor derived from spleen cell conditioned medium is present in the culture (Axelrad et al., '78; Iscove and Guilbert, '78).

Recent studies suggest that the 8 day BFU-E, the 3 day BFU-E and the CFU-E represent different stages in the erythropoietic differentiation pathway. Gregory and Henkelman ('76) surveyed the number of CFU-S, 8 day BFU-E, 3 day BFU-E, CFU-E and nucleated cells in individual spleen colonies assuming a positive correlation between the numbers of any two classes of cells within a colony indicates a close relationship between them. Significant positive correlations were found between CFU-S and 8 day BFU-E, between the 8 day BFU-E and the 3 day BFU-E, between the 3 day BFU-E and CFU-E and between the CFU-E and the nucleated cells. Correlations between other cell class (CFU-S and CFU-E, etc) were very weak or nonexistent, suggesting that the cells are found in the differentiation pathway in the following order:

CFU-S-->8 day BFU-E-->3 day BFU-E-->CFU-E-->erythrocytes.

Studies showing that cells from 8 days burst give rise to both 3 day bursts and 48 hour colonies upon transfer to secondary cultures is further evidence in support of this differentiation sequence (Gregory and Henkelman, '76).

The most logical interpretation of these observations is that one of the BFU-E populations represents the committed progenitor cells for the erythroid line of differentiation. Recently, megakaryocytes have been found in the 8 day bursts, indicating that the 8 day BFU-E, similar to the GM-CFC, is bipotential (McLeod et al., '76, Humphries et al., '79).

3) Progenitors From Other Blood Cell Classes

a) Eosinophil Progenitor Cells

Eosinophil colonies were first detected in cultures of human marrow (Iscove et al., '71) and peripheral blood (Chervenick and Boggs, '71), however, they have since been found in mouse cell cultures as well (Metcalf et al., '74). When mouse bone marrow cells are cultured in semisolid agar in the presence of a suitable inducing factor, eosinophil colonies become visible at 4 days of incubation and increase in size progressively so that by 2 weeks some contain 5000 or more cells. The eosinophil colony stimulating factor (EO-CSF) is derived from media conditioned by spleen or lymph node cells cultured with pokeweed mitogen and appears to be different from the factor responsible for stimulating the granulocyte-macrophage colonies (GM-CSF) (Metcalf et al., '74; Metcalf et al., '78).

Morphologically, the eosinophil colonies are quite uniform, consisting of loose globular aggregates of

translucent cells. Most 7 day colonies consist of immature cells with basophilic cytoplasm, while the more mature colonies contain cells which show definite signs of eosinophilic differentiation (acquisition of eosinophilic granules and Fc receptors) (Metcalf et al., '74; Rabellino and Metcalf, '75).

Little is known about the eosinophil colony forming cells (EO-CFC) although velocity sedimentation experiments suggest that they are slightly smaller than the GM-CFCs. In addition to their presence in the bone marrow, EO-CFCs are found in the spleen, fetal liver, and spleen colonies derived from adult marrow CFU-s (Metcalf et al., '74). The EO-CFCs most likely represent committed progenitor cells, since in all cultures only pure eosinophil colonies are found.

b) Megakaryocyte Progenitor Cells

Megakaryocyte colonies develop in the same culture system that promotes the growth of eosinophil colonies (Metcalf et al., '75a). Development of the megakaryocyte colonies is dependent upon the presence of megakaryocyte colony stimulating factor (MEG-CSF) obtained from the same source as the EO-CSF. Whether these two factors are identical remains to be determined.

Colonies first become visible at 3-4 days of culture and their growth is progressive until 7 days. Two types of

colonies have been observed in cultures of mouse bone marrow cells: (1) small loose aggregates of up to 40 large cells that appear to be megakaryocytes, and (2) loose colonies of up to 120 large cells, often with a tight central core surrounded by a more disperse region of cells of varying sizes. Cells within the colonies appear to be rich in acetylcholinesterase and react with antiplatlet serum, indicating that they are megakaryocytes. Fully mature cells with platelet shedding ability are rarely found in these cultures. More recently, McLeod et al., ('76) described the development of megakaryocyte colonies in plasma gel cultures stimulated with plasma from anemic sheep. The cells within these colonies exhibit full cytoplasmic maturation and extensive platelet formation.

Since a linear relationship exists between the number of megakaryocyte colonies and the number of cells cultured, it is assumed that the colonies are derived from a single megakaryocyte colony forming cell (MEG-CFC) (Metcalf et al., '75a). Both the sedimentation velocity (4.2 mm/hr) (Metcalf et al., '75a) and the buoyant density (1.075 g/cc^3) (Nakeff, '76) indicate that MEG-CFCs are relatively small cells, similar to the GM-CFCs and the EO-CFCs. As with the eosinophil colonies, megakaryocyte colonies are usually pure suggesting that the MEG-CFCs are committed progenitors.

c) Lymphoid Progenitor Cells

B-lymphocyte colonies develop in agar cultures of spleen, lymph node or peripheral blood cells if 2-mercaptoethanol is included in the medium (Metcalf et al., '75b). Though some cell maturation does take place within the colonies, it is doubtful whether the cell initiating them, the B-lymphocyte colony forming cell (BL-CFC), is a primitve progenitor. Rather, it most likely represents a relatively differentiated member of the B-lymphocyte class, since most of the representatives are recirculating cells that express both immunoglobulin and Fc receptors (Johnson et al., '76; Metcalf et al., '76).

Colonies of T-lymphocytes were first detected in agar cultures of mouse thymic lymphocytes (Bujadoux et al., '72). Since then, several methods have been developed for the growth of human T-lymphocyte colonies (Rozenszajn et al., '75; Claesson et al., '77). As with the B-lymphocyte colonies, it appears as if the cells initiating these colonies are relatively mature since most of them are the size of small lymphocytes and a large majority form sheep red blood cell rosettes - a characteristic of relatively mature human T-lymphocytes.

In summary, most of the in vitro assays discussed appear to detect a population of hemopoietic precursor cells, more differentiated than the CFU-S, and restricted to one or two lines of differentiation.

E. Developmental Aspects of Hemopoiesis

1) Hemopoietic Organs

In mammals and birds, hemopoiesis is first observed in the yolk sac mesoderm. In the chick embryo, foci of erythropoiesis can be observed within 2 days of incubation in primitive blood islands associated with the mesoderm (Reviewed in Romanoff, '60; Lucas and Jamroz, '61; Metcalf and Moore, '71), while both granulopoiesis and thrombopoiesis are found shortly thereafter (Edmonds, '66). GM-CFCs can be detected in the yolk sac by 12 days of incubation (Dodge and Moscovici, '73). The hemopoietic activity of the yolk sac reaches a peak between 10-14 days incubation and then begins to decline, ceasing prior to hatching. Primitive blood islands in the mouse embryo develop in the yolk sac mesoderm by 7 1/2 days gestation (Metcalf and Moore, '71). Unlike the situation in the chick, the mouse yolk sac is a hemopoietic organ for a relatively short period of time (day 7 - day 12 of gestation) during fetal development. Furthermore, the hemopoietic activity appears to be restricted to erythropoiesis, although both CFU-S and GM-CFCs can be detected at this time. Lymphopoiesis is not present in either the chick or mouse yolk sac, however, both contain cells capable of repopulating the lymphoid organs of irradiated hosts (Moore and Owen, '67b; Tyan, '68; Tyan and Herzenberg, '68; Moore and Metcalf, '70).

In the mouse embryo, hemopoietic activity appears to shift from the yolk sac to the developing fetal liver. Erythropoietic activity is initially observed in the liver between 10 and 11 days gestation, a time when the yolk sac hemopoiesis begins to decline (Reviewed in Metcalf and Moore, '71). Megakaryopoiesis and granulopoiesis follow shortly thereafter, however erythropoiesis is by far the most prominent activity found in the liver. CFU-S and GM-CFCs are found at the onset of liver hemopoiesis and they are present at relatively high numbers throughout fetal development. The degree of hepatic hemopoietic activity, including the number of CFU-S and GM-CFCs, declines to undetectable levels during the first few weeks after birth (Silini et al., '67; Metcalf and Moore, '71). Unlike the mouse, the chick embryo liver supports very little hemopoiesis (Romanoff, '60).

The spleens of both the chick and mouse embryo are hemopoietic organs and both pass through similar phases of development. An initial, but brief, erythropoietic phase (9-10 day in chick, 15 day in mouse) is followed by a much more extensive granulocytic phase (11-18 day in chick, 17-20 day in mouse). Shortly before hatching in the chick, and at birth or shortly thereafter in the mouse the spleen undergoes a transition from a myelopoietic to a lymphoid organ - a status it maintains throughout normal adult life (Romanoff, '60; Lucas and Jamroz, '61; Metcalf and Moore, '71). CFU-S and GM-CFCs can be detected in the mouse spleen

by 15 days gestation, however, maximum numbers are not reached until 3 weeks after birth (Metcalf and Moore, '71). Following this peak, both cell populations decline to the relatively low levels found in the adult spleen. GM-CFCs have been found in the chick embryo spleen at 14 days of incubation (Keller et al., '79).

The bone marrow, which becomes the major hemopoietic organ in the adult, shows signs of erythropoiesis and granulopoiesis by 11-12 days incubation in the chick while in the mouse, only granulopoiesis is observed in fetal life and this is not detected until 17-18 days gestation (Metcalf and Moore, '71). Both CFU-S and GM-CFCs are present in the fetal marrow, although at relatively low levels. Increase in hemopoietic activity (including the number of CFU-S and GM-CFCs as well as the onset of erythropoiesis in the mouse marrow) is seen shortly after birth or hatching, concomitant with a decrease in the hemopoietic activity of all the other organs.

The thymus is primarily a lymphopoietic organ, although there is some evidence that it supports granulopoiesis early in development in the chick (Romanoff, '60). Lymphopoiesis becomes prominent in the chick embryo thymus between 10 and 12 days incubation while the fetal mouse thymus shows signs of lymphoid development by 13 days gestation (Metcalf and Moore, '71). Very few CFU-S or GM-CFCs are found in the developing or adult mouse thymus.

The bursa of Fabricius in the chick embryo initially supports a considerable amount of granulopoiesis, however, by 15-16 days incubation, lymphopoiesis becomes the predominant line of hemopoietic activity (Romanoff, '60, Metcalf and Moore, '71).

2) Origin of Hemopoietic Cells

The origin of the hemopoietic cells in the different organs has been a controversial issue amongst hematologists for the past 100 years. Though the most heated debates surrounded the origin of the lymphocytes in the thymus, similar arguments pertain to the origin of the hemopoietic cells in the other organs.

A number of theories was put forward describing the lymphoid development of the thymus. The two most popular were the transformation theory proposed by Kolliker and the substitution theory initially formulated by Hammar (Reviewed in Metcalf and Moore, '71). The transformation theory states that lymphocytes originate by transformation of the epithelial cells in the early thymic anlage, while the substitution theory proposes that lymphocytes are derived from surrounding undifferentiated mesenchyme and then migrate into the early presumptive thymus. A third theory, the hematogeneous theory, is basically an extension of the substitution theory and states that the precursors of lymphocytes migrate into the early thymus from the

circulation, not from the surrounding mesenchyme (Metcalf and Moore, '71). Though both the transformation and substitution theories received support from the early hematologists neither could be proved nor disproved since acceptance of either theory was based on the interpretation of morphological studies.

Renewed interest in the origin of the hemopoietic cells came with the introduction of a variety of new cell markers, tissue culture methods and microscopic techniques.

Support for the transformation theory was provided by Ackermann and Knouff ('59, '64, '65) who, through a series of electron microscopy studies, described transitional forms between epithelial cells and lymphoid cells in both the bursa of Fabricius and the thymus. These studies, unfortunately, suffer from the same criticism as those of the earlier hematologists in that they attempt to interpret static morphological sequences in dynamic terms.

Using an improved culture system, Auerbach ('61) found that thymic epithelium from 12-12 1/2 days mouse embryo becomes lymphoid in vitro, provided it is cultured in close association with mesenchyme. This mesenchymal influence appears to be humoral since it can be mediated across a cell impermeable membrane. The interpretation of these results, that the lymphocytes arise by direct transformation of the epithelial cells, was based on the assumption that the epithelial anlage of the 12-12 1/2 day mouse embryo is pure,

containing no primitive hemopoietic cells.

More recent evidence, favouring the transformation theory comes from a series of experiments by Turpen and Volpe ('73) and Turpen et al. ('75). At a very early stage of development (before circulation is established) presumptive thymuses were exchanged between diploid and triploid frogs, the cells of which can be differentiated on the basis of their DNA content. Following maturation of the frogs, these investigators found that more than 95% of the thymocytes in the donor thymus, 15% of the kidney cells, 30% of the spleen cells and 43% of the bone marrow cells are derived from the donor graft. Since the grafts were exchanged prior to the establishment of a circulatory system, it was assumed that they contain no extrinsic hemopoietic cells, and thus these findings were interpreted in favour of a transition of epithelial cells to lymphocytes.

Although the transformation theory was doubted by some of the early hematologists, it was the more recent cell marker studies that severely challenged the concept of transformation of epithelial cells into lymphocytes. The first of such studies was that of Moore and Owen ('65) who employed the chicken sex chromosome as a marker for tracing cellular traffic in the chick embryo. The blood vessels of young embryo were joined through parabiosis (chorioallantoic membrane or yolk sac), and in situations where partners were

of different sexes, the hemopoietic organs were tested for cellular chimerism. In some combinations, up to 50% of the cells in the bone marrow, bursa, and spleen in each embryo were derived from the opposite partner following parabiosis by the chorioallantoic (CAM) membrane. Chimerism in the thymus was observed only when the embryos were parabiosed by the yolk sac (Moore and Owen, '67a). These observations, that a relatively high degree of chimerism was established in most of the hemopoietic organs tested, strongly suggest that cells within these organs are derived from an influx of blood borne hemopoietic stem cells and not from an in situ transformation of epithelial cells, and thus support the hematogenous theory. Further support for this theory was provided by organ grafting experiments in which it was shown that embryonic thymus or bursa grafted to the CAM become populated, almost exclusively, by host hemopoietic cells (Moore and Owen, '66, '67a). The first cells to enter the grafts are heavily basophilic cells with prominent nucleoli. Since similar cells are also found in normal stages of thymic development prior to the onset of lymphopoiesis, it was postulated that they represent an influx of hemopoietic stem cells from the circulation. Finally, in a subsequent study, it was shown that the hemopoietic organs of the chick embryo contain stem cells capable of repopulating irradiated hosts, an observation that provides further support for the hematogeneous theory (Moore and Owen, '67b). Since the yolk sac is the earliest developed organ with the capacity to

repopulate irradiated chick embryos, it was thought that hemopoietic stem cells originate here and then migrated to the intraembryonic organs throughout development. This idea has been further supported by Moore and Metcalf ('70) who found that cultures of whole mouse embryos contain hemopoietic cells, only if the yolk sac is present. Removal of the yolk sac prior to culturing, results in the development of embryos without blood cells. Furthermore, they noted that normal numbers of GM-CFCs develop only in cultures of mouse embryos with yolk sacs. No detectable colony forming cells were found in mouse embryos cultured without the yolk sac, while yolk sacs cultured alone contain greater than normal numbers of GM-CFCs. These findings, along with those showing a rise in the number of blood borne CFU-S and GM-CFCs prior to the onset of hemopoiesis in the fetal liver, strongly suggest that the development of intraembryonic hemopoiesis is dependent upon an influx of stem cells from the yolk sac.

Keeping in mind the results from the parabiosed chicks, Owen and Ritter ('69) investigated the development of both chick and mouse embryonic thymus by culturing them in millipore chambers on the chick embryo CAM. They found that 8 and 9 day chick embryo thymic rudiments, that contain large numbers of basophilic cells with prominent nucleoli, show considerable lymphoid development in culture. Rudiments from 6 day embryos, on the other hand, fail to develop any lymphopoietic activity in culture. Similarly, 12 day mouse

thymus rudiments also containing the basophilic cells develop quite normally in culture, while the 11 day rudiments, with very few basophilic cells, show no lymphopoiesis when cultured for 6 days. These results suggest that lymphopoiesis in the thymus is initiated by large basophilic cells (stem cells) that most likely migrate to the thymic rudiment from the circulation. Furthermore, they show that the 12 day mouse thymic rudiments, used in the cultures by Auerbach, already contain such cells.

Perhaps the most convincing evidence in support of the hematogenous theory was provided by the studies of Le Douarin and Jotereau ('73, '75) and Houssaint et al. ('76). Using the difference in the degree of dispersedness of the chromatin between Japanese quail cells and chick cells as a marker, they found, through a series of elegant heterospecific grafting experiments, that the hemopoietic cells in all the organs are derived from an extrinsic source. Since the grafting was carried out at the early stages of organ development, a precise time of colonization could be determined. By grafting chick thymus tissue of various ages into the somatopleure of 3 day quail hosts and vise versa, it was found that the chick thymic rudiment is initially invaded by large basophilic cells with prominent nucleoli between 6.5 and 8 days of development (Le Douarin and Jotereau, '75). The quail rudiment receives its influx of cells between 5 and 6 days incubation. A second wave of immigrating cells appears to colonize the thymus of both

species shortly before hatching. Colonization of the bursa by the basophilic cells occurs between 7 and 11 days incubation in the quail and between 8 and 14 days incubation in the chick (Houssaint et al., '76).

More recent studies have shown that, although the chick thymus is not normally colonized before 6.5 days incubation, colonization can occur at a later stage provided the rudiment has not already been populated by stem cells (Le Douarin, '78). Colonization of the thymic anlage appears to be controlled by a chemotactic factor produced by the epithelium of the thymus. Production of this factor might be controlled by the number of stem cells in the thymus since the organ appears to lose its "attractiveness" for these immigrating cells following complete colonization. These findings not only conclusively demonstrate that the hemopoietic cells are derived from blood borne precursors, they also suggest that the colonization of the thymus (and probably most of the other hemopoietic organs) by these cells occurs at a very precise time during development.

Using this same cell marker, Dieterlen-Lievre ('75), Martin et al. ('78) have recently challenged the yolk sac origin of the hemopoietic stem cell, initially proposed by Moore and Owen. Whole quail embryos (without yolk sac) were transplanted onto chick embryo yolk sacs at a very early stage of development prior to the establishment of a circulatory system. Embryos that survived the operation were

found to have their thymus and bursa populated almost entirely by donor quail cells. In approximately one half of the spleens tested, some chicken cells of yolk sac origin were detected. This appeared to be a transitory colonization, since the number of chicken cells begins to diminish by 13 days of development. Similarly, chick cells were found in some of the bone marrows tested, however, in the majority of cases, quail cells predominated. This intraembryonic cell colonization was not due to inaccessability of the chicken cells to the organs, since, in the early stages, more than 95% of the circulating erythrocytes were of chick origin. Surprisingly, it was found that the early yolk sac (at the time of grafting) does contain cells capable of populating the lymphoid organs, however, in these experiments they do not appear to play a major role in this respect (Le Douarin, '78). Perhaps one of the more interesting findings was that, in some of the older chimeras, the stem cells appear to migrate from the embryo and colonize the yolk sac (Martin et al., '78), an observation contrary to the hypothesis forwarded by Moore and Owen. If the yolk sac was taken at this time and used in repopulation studies, it would appear as if it is the ultimate source of stem cells. This may explain the apparent discrepancy between these findings and those of Moore and Owen. As the chimeras became older, quail hemopoiesis became more predominant, and in some cases, all the yolk sac erythropoietic cells were of donor origin. One criticism of

this work is that the graft crosses a species barrier and this may affect the migratory pattern of the hemopoietic stem cells. Similar results were obtained from experiments in which chick embryos were grafted onto chick yolk sacs, suggesting that the origin of the stem cells which colonize the hemopoietic organs of the chick embryo is intraembryonic (Lassila et al., '78).

In addition to the above experiments with the chick embryo, recent reinvestigation of thymus development in amphibians has shown that presumtive thymus grafts do become populated by host lymphocytes, thus bringing these results in line with the hematogenous theory as well (Turpen et al., '77, Volpe et al., '77).

It appears, therefore, that little controversy remains over the development of the thymic lymphocytes, with most studies now strongly supporting an extrathymic origin for these cells.

F. Regulation of Hemopoiesis

1) Humoral Regulators

a) Erythropoietin

Regulation of erythropoiesis by a humoral factor was first proposed by Carnot and Deflandre who observed that plasma from rabbits made anemic by bleeding increase peripheral red cell counts when injected into normal recipients. This early observation has since been repeated

many times in different species and it is now widely accepted that a humoral regulator for erythropoiesis does exist (Reviewed in Krantz and Jacobson, '70; Metcalf and Moore, '71; Gordon, '73).

The factor, known as erythropoietin (EPO), has been purified from two different sources (human urine and anemic sheep serum) and, although in each case the molecule is a neuraminic acid containing glycoprotein, it appears to differ in size depending on the source (human EPO M.W. 39,000 and sheep EPO M.W. 46,000) (Goldwasser and Kung, '72; Miyake et al., '77). In adults, the principle site of production of EPO appears to be the kidneys; however, in some species, external sources have been detected following nephrectomy (Krantz and Jacobson, '70; Gordon, '73). Controversy exists as to whether the kidney actually produces EPO or an enzyme (erythrogenin) that converts a serum substrate to EPO. The principle factor regulating the level of EPO is considered to be oxygen tension that is mediated directly by the number of red blood cells. Thus, manipulation such as bleeding or subjection to an hypoxic environment will result in an oxygen deficiency and an increase in serum EPO levels. Hypertransfusion, on the other hand, will increase the oxygen carrying capacity of the blood, and therefore result in decreased EPO levels. The function of EPO, once released into the blood, is to: (1) stimulate the differentiation of recognizable erythroid cells from unrecognizable precursors, (2) effect the release

of red blood cells into the circulation, (3) increase haeme synthesis in bone marrow cells and (4) shorten the cell cycle time of maturing red cells. At the molecular level, EPO very quickly initiates both DNA dependent RNA and protein synthesis. DNA synthesis follows and cell proliferation begins within several hours (Krantz and Jacobson, '70; Gordon, '73).

Although some studies indicate that EPO acts directly on the CFU-S, (Goldwasser, '75; Van Zant and Goldwasser, '77b; Van Zant et al., '79), most evidence suggests that the target cell for EPO is more differentiated than the pluripotent stem cell. It has been shown that the cells which respond to erythropoietin (the erythropoietin responsive cell - ERC) and the CFU-S differ with respect to size (Stephenson and Axelrad, '71), mitotic activity (Lajtha et al., '69) and responsiveness to hypoxia (Bruce and McCulloch, '64). In addition, it has been found that suppression of EPO production (by hypertransfusion) in irradiated mice with regenerating hemopoietic systems has no effect on the number of nonerythroid spleen colonies (Schooley, '64; Curry et al., '67). Such manipulations do, however, reduce the large erythroid colonies to small aggregates of erythropoietin responsive cells which are most likely derived from the CFU-S by an EPO independent phase of differentiation (Schooley, '66; Curry et al., '67).

Two ESC, the BFU-E and the CFU-E, have been cloned in

vitro. The characteristics of these cells suggests that the population of cells responding to EPO is heterogenous and may represent a large number of cells at various stages of differentiation along the erythropoietic pathway. That EPO does not dramatically affect other types of in vitro colonies is additional evidence that the target cell is not a pluripotent cell (Metcalf and Johnson, '79).

Studies on fetal erythropoiesis have shown that it is also regulated by EPO. Zanjani et al. ('74) noted an increase in EPO levels within 6 to 15 hours of bleeding a fetal goat. This increase was found regardless of whether the fetus or the mother was nephrectomized, suggesting that, at this stage of development, the kidney is not the primary source of EPO. Recent studies showing that the fetal liver produces EPO in vitro suggests that this organ might be the main EPO producer during fetal development (Zucali et al., '77). Although it was initially thought that yolk sac erythropoiesis was refractory to EPO (Cole and Paul, '66), more recent studies demonstrating that EPO stimulates haem synthesis in cultures of disaggregated cells from whole 8-day embryos and yolk sac tissue suggests that differentiation of the early erythroid cells is regulated by mechanisms similar to those regulating adult erythropoiesis (Bateman and Cole, '71).

b) Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)

Granulocyte-macrophage colony stimulating factor (GM-CSF) also known as colony stimulating activity (CSA) and macrophage and granulocyte inducer (MGI), stimulates the development of granulocyte and macrophage colonies in vitro (Metcalf, '77). The target for GM-CSF is most likely the committed progenitor granulocyte-macrophage colony forming cell (GM-CFC), since the factor does not stimulate the development of any other type of colony (erythroid, eosinophilic, megakaryocytic, B-lymphoid, or mixed) although it has recently been shown that it can promote the survival in vitro of several other progenitor cells (Metcalf, '77; Metcalf et al., '79). GM-CSF is required for the survival of GM-CFCs in culture and a number of observations suggest that it is more than a nutritional factor: (1) GM-CSF can force noncycling GM-CFCs to enter S phase of the cell cycle within 3 hours (Moore and Williams, '73a), (2) GM-CSF has an effect on the cell cycle length of the dividing colony cells (Metcalf and Moore, '73), the lag phase before initiation of proliferation in GM-CFCs (Metcalf, '70) and on the growth rate of the colonies (Robinson et al., '67a), and finally (3) GM-CSF initiates RNA synthesis in granulocytic cells within 10 minutes (Burgess and Metcalf, '77). Whether GM-CSF provides a differentiation stimulus is a question remaining to be answered.

GM-CSF can be extracted from a large variety of tissues such as submaxillary gland, lung, thymus, kidney, spleen, lymph nodes, pancreas, yolk sac and marrow (Reviewed in Metcalf and Moore, '73; Metcalf, '77). Colony stimulating factor is also produced and/or released by a variety of cells in culture including lymphocytes, L-cells, monocytes, macrophages, fibroblasts, lung cells, bone marrow stromal cells and whole embryo cells. Other sources of the factor include serum from endotoxin treated animals and human urine.

Most of the GM-CSFs fall into 2 broad classes with respect to the types of colonies they stimulate (a) those that stimulate a large proportion of granulocyte colonies if used in high concentration and, (b) those that stimulate only macrophage colonies regardless of the concentration used (Metcalf, '77, '78). One interpretation of this general observation is that the GM-CSF's of the first class influence the progenitor cells to differentiate along a granulocytic pathway, while those in the second group favour differentiation into macrophages. Recent findings, showing that the small GM-CFCs generate more granulocyte colonies while the larger ones initiate more macrophage colonies, suggests that the different GM-CSFs might act on different target cells in the progenitor cell compartment (Metcalf and MacDonald, '75).

Purification of the factor derived from mouse lung

tissue or L-cells has revealed that GM-CSF is a neuraminic acid containing glycoprotein with different molecular weights depending on the source (23,000 for mouse lung GM-CSF and 70,000 for L-cell GM-CSF) (Burgess et al., '77; Stanley and Heard, '77).

The major factor influencing GM-CSF levels is thought to be exposure to bacterial antigens and other bacterial products. Thus, injection of polymerized flagellar antigen or bacterial endotoxin results in a rise in tissue levels of GM-CSF within 30 minutes, reaching peak levels by 3-6 hours (Sheridan and Metcalf, '72). The rise in tissue GM-CSF levels is paralleled by a rise in the level of serum GM-CSF, sometimes reaching concentrations 50-100 times higher than normal (Metcalf, '71a). Whole body irradiation (Hall, '69), some tumors and leukemias (Metcalf and Foster, '67a; Metcalf et al., '69; Robinson et al., '67a; Robinson, '74), and injection of antineutrophil serum (Shadduck and Nagabhushanam, '71), will also elevate the levels of serum GM-CSF.

Though a large majority of the studies on GM-CSF have been carried out in vitro, there is some evidence suggesting that this factor(s) is an in vivo regulator of granulocyte and macrophage production. In both normal as well as a number of disease states, one can observe related fluctuation between GM-CSF levels and granulopoiesis (McNeil, '70; Metcalf, '71a,c; Metcalf and Stevens, '72;

Dale et al., '71; Shadduck and Nagabhushanam, '71; Gatti et al., '73; Trudgett et al., '73; Chan and Metcalf, '73; Guerry et al., '74; Moore et al., '74). Injection of GM-CSF produces no immediate increase in the level of blood GM-CFCs, indicating that the factor does not cause a release of the prognitor cells into the circulation (Metcalf, '74). Injection of GM-CSF over a 6 day period does, however, produces a substantial rise in spleen and blood GM-CFC levels, as well as a rise in peripheral blood neutrophil levels (Bradley et al., '69). Although these studies are not conclusive, they strongly suggest that GM-CSF plays some physiological role in the regulation of both neutrophil and macrophage production.

c) Regulators For The Other Lines of Hemopoiesis

Thrombopoietin, a factor that increases blood platelet levels, has been detected in the serum of normal and thrombopenic animals and humans (Reviewed in Abildgaard and Simone, '67; Cooper, '70; Metcalf and Moore, '71). Kelemen et al., ('58) showed that a single injection of thrombopoietin would substantially increase blood platelet levels 3 to 4 days later. The amount of thrombopoietin found in the serum is most likely controlled by blood platelet numbers, since a loss of these cells through bleeding increases the level of the factor while transfusion with platelets depresses further platelet production (De Gabriele and Penington, '67). Other treatments found to increase

plasma thrombopoietin levels include X- or UV irradiation, splenectomy and injection of antiplatelet serum. Although chemically, it is similar to EPO and GM-CSF (heat labile, non-dialysable glycoprotein), thrombopoietin appears to be a separate factor, since in certain disease states it is found at a high concentration while EPO levels are normal (Schulman et al., '60). Although the site of production of this factor is not known, recent studies suggest that the kidney might be involved (McDonald, '75).

Other studies have shown that a factor derived from 2 mercaptoethanol or pokeweed mitogen stimulated mouse lymphocytes initiates the growth of megakaryocyte colonies in vitro (Metcalf et al., '75a). Production of this megakaryocyte colony stimulating factor (MEG-CSF) requires an interaction between splenic lymphocytes and adherent cells (Metcalf and Johnson, '78). That spleen cells from the nu/nu congenitally athymic mice fail to liberate any detectable amounts of this factor suggests that T-lymphocytes are involved in its production. At the present time, it is not known whether MEG-CSF (glycoprotein, M.W. of 40,000) is different from GM-CSF also found in the spleen cell conditioned medium. Furthermore, the relationship between MEG-CSF and thrombopoietin remains to be determined.

Factors stimulating eosinophil production in vivo have been found in animals suffering from parasitic infection. Basten and Beeson ('70) found that large lymphocytes

(thoracic duct) from Trichinella infected rats elaborate a factor capable of stimulating eosinophil production in normal rats. Other studies have shown that lymphocytes from Trichinella infected mice produce a factor which stimulate eosinophilopoiesis in vitro (Ruscetti et al., '76). Recently, it has been shown that pokeweed mitogen stimulated lymphocytes that produce MEG-CSF also produce an eosinophil colony stimulating factor (EO-CSF) capable of initiating the formation of eosinophil colonies in agar cultures (Metcalf et al., '74; Metcalf and Johnson, '78). EO-CSF, a heat labile, non dialyzable protein, with a molecular weight of approximately 40,000-50,000 can be separated from GM-CSF by starch gel electrophoresis. It has not, however, been separated completely from MEG-CSF (Metcalf et al., '74; Metcalf et al., '78). Again, it is not known whether EO-CSF is the same as the factor derived from the lymphocytes of the parasite infected animals.

Although a number of reagents and factors are capable of potentiating B-lymphocyte and T-lymphocyte colony growth in vitro, a lymphocyte colony stimulating factor equivalent to EPO or CSF, has not been discovered. There are, however, several factors capable of initiating the differentiation of pre-T and pre-B cells in vitro.

Thymopoietin, a small protein (M.W. 5562) derived from thymic epithelial cells, initiates the appearance of TL and Thy-1 antigens on bone marrow and spleen cells (Basch and

Goldstein, '74, '75a,b; Schlesinger and Goldstein, '75; Scheid et al., '75; Goldstein, '75; Goldstein et al., '76). The target appears to be a cell committed to T-cell differentiation, since B-cells, CFU-S and erythropoietin sensitive cells are not affected. The fact that the differentiation process initiated by thymopoietin occurs very quickly and without cell division further supports the notion that the target cell is a relatively mature cell, committed to the T-lymphocyte line of hemopoiesis (Storrie et al., '76).

Bursapoietin, a factor obtained from the chicken bursa, appears to be involved at some stage in the differentiation pathway of B-lymphocytes (Brand et al., '76; Goldstein et al., '76). Though the action of bursapoietin is not entirely confined to B-lymphocyte differentiation, its main activity appears to be the induction of the expression of immunoglobulin molecules and B-lymphocyte antigens (BU-1) (Gilmour et al., '76) on pre-B cells. The in vivo relevance of thymopoietin and bursapoietin is presently unknown.

d) Humoral Regulators of the CFU-S

The regulators discussed thus far appear to act at the level of the committed progenitor cell, although, there is now evidence for several classes of factors that interact directly with the pluripotent stem cell.

Marrow cells from phenylhydrazine (PHZ) treated,

hydroxyurea treated or irradiated mice produce a factor that stimulates quiescent CFU-S into cycle (Frindel et al., '76; Lord et al., '76; Wright and Lord, '77, '78). A similar stimulatory factor can be derived from fetal liver cells. Factors such as this might be responsible for the observed onset of proliferation of marrow CFU-S following sublethal irradiation (Croizat et al., '70). Factors that induce the proliferation of CFU-S have also been obtained from cultures of fibroblasts (Lowenberg and Dickie, '77). Whether fibroblasts produce similar factors in vivo remains to be determined. Though the nature of this fibroblast-derived factor is not known, it has been shown to be different from GM-CSF.

In addition to factors affecting the cell cycle status of the CFU-S, there is now evidence suggesting that some of the early differentiation stages in hemopoiesis may be controlled by long range humoral regulators, unrelated to EPO or CSF. For instance, the number of BFU-E or CFU-S does not change dramatically following manipulations that are known to alter the in vivo EPO levels (Iscove, '77; Wagemaker, '76). A significant increase in the number of CFU-S and BFU-E in cycle is observed, however, in regenerating spleen and marrow. In addition, it has been shown that BFU-E, but not CFU-E can survive in culture for several days in the absence of any detectable EPO, provided that the system contains sufficient concentrations of serum (Iscove and Guibert, '78). Addition of EPO 4 or 5 days later

results in the formation of large bursts containing erythroid cells. On the other hand, cultures containing EPO, with only small amounts of serum, promote the survival of CFU-E but not BFU-E. The interpretation of these findings is that the serum contains a burst promoting activity (BPA) required for the differentiation of the BFU-E, to a stage at which the cells become sensitive to EPO. The implication of this interpretation is that the BFU-E is a relatively undifferentiated cell, quite closely related to the CFU-S. Recent studies showing that BFU-E and CFU-S have identical physical properties (Referred to in Wagemaker, '78), as well as those demonstrating that in vitro bursts contain cells capable of generating in vivo spleen colonies (Humphries, '79) support this concept. In addition to its presence in serum, BPA has also been detected in medium conditioned by Pokeweed Mitogen stimulated spleen cells (Iscove and Guilbert, '78).

Wagemaker ('78) has found a similar burst forming activity (BFA) associated with irradiated bone marrow cells, in mouse serum and in human leukocyte conditioned medium (HLCM). Addition of irradiated bone marrow cells to BFU-E cultures results in an increased number of bursts as well as an increased sensitivity of the BFU-E to EPO. The marrow cells responsible for this BFA activity were found to have a density of 1.083 g/cm^3 and a sedimentation velocity profile showing two peaks, one at 4.7 mm/hr and a second one at 6.1 mm/hr. A BFA-like activity was also detected in the serum of

ex-hypoxic polycythemic and phenylhydrazine treated mice. The presence of this activity in the ex-hypoxic mice coincides with a slight, but significant, rise in the number of marrow BFU-E in these mice, indicating that perhaps this burst forming activity plays some in vivo role in regulation (Wagemaker et al., '76).

In addition to the irradiated marrow and mouse serum, human leukocyte conditioned medium (HLCM) was found to contain a factor or factors that enhance both the number of bursts and the number of granulocyte and macrophage colonies in vitro (Wagemaker, '78; Wagemaker and Peters, '78). This activity affecting the burst number could be identical to the BFA associated with the irradiated marrow cells, since when present in culture together, their effects are non additive. The action of HLCM, in enhancing more than one type of colony suggests that the factor(s) may be acting on a primitive cell population, perhaps the CFU-S. The fact that HLCM initiates DNA synthesis in CFU-S is consistent with this interpretation (Wagemaker and Peters, '78).

Along these same lines, Johnson and Metcalf ('77) have recently described a biological activity found in pokeweed mitogen spleen cell conditioned medium that will initiate the development of mixed hemopoietic colonies in fetal liver cultures. The mixed colonies were shown to be clones and to contain cells capable of initiating in vivo spleen colonies, indicating that they are most likely derived from a

pluripotent stem cell (Metcalf et al., '79). In this particular system, the factor in the conditioned medium appears to act directly on the stem cell. Whether the factor that initiates the mixed hemopoietic colonies is separate from the other humoral regulators (GM-CSF, MEG-CSF, EO-CSF, and E-CSF) present in the conditioned medium remains to be determined.

Similar mixed hemopoietic colonies have recently been detected in cultures of human marrow and peripheral blood (Fauser and Messner, '78, '79). In this system, the development of the mixed colonies is dependent upon the presence of both erythropoietin and medium conditioned by phytohemagglutinin stimulated leukocytes. Again, the implications are that the conditioned medium contains a factor that acts at the pluripotent stem cell level.

In summary, there is a considerable body of evidence suggesting that factors do exist that affect the early differentiation stages of hemopoiesis. The physiological significance of these factors and their relationship to the factors described in the previous section remains to be determined.

e) Inhibitors of Hemopoiesis

It is obvious that a continuously regenerating system such as the hemopoietic system must be under strict regulation; both in the positive and negative sense.

Unfortunately most of the studies on negative regulation have failed to conclusively demonstrate the existence of physiologically active inhibitors of hemopoiesis. Nonetheless, there are a number of observations suggesting that such inhibitors do exist.

It has been shown that the serum of normal mice and humans contains a material capable of inhibiting granulocyte-macrophage colony formation in vitro (Metcalf and Foster, '67a; Chan and Metcalf, '70; Chan, '71; Mortensen et al., '79). The fact that this material is sensitive to chloroform treatment (Chan et al., '71; Granstrom, '72), that it can be separated from the serum by floatation centrifugation over sodium chloride (Granstrom, '72; Metcalf and Russell, '76) and that it reacts with antihuman B-lipoprotein (Granstrom, '72) indicates that it is a lipid-containing molecule. The in vivo significance of this inhibitory substance is, at present, a controversial issue. Cultures containing dilute concentrations of the inhibitor tend to support the development of considerably more macrophage colonies than is normally observed indicating that this lipid-containing material may play some regulatory, rather than inhibitory, role in vivo (Chan, '71; Metcalf and Russel, '76). Other studies, showing a depressed level of the inhibitor in certain leukemic states as well as in the serum of mice undergoing hemopoietic regeneration, suggest that this molecule may, in fact, be a physiological inhibitor of hemopoiesis (Chan and Metcalf, '70, '73; Chan,

'71; Metcalf et al., '71a). On the other hand, the lack of target cell specificity displayed by the inhibitor (Metcalf and Russel, '76), its high concentration in normal serum (Beran,'74; Metcalf and Russel, '76) and the lack of correlation between concentration of the inhibitor and the different phases of granulopoiesis (Metcalf, '77), suggest that it might represent an in vitro artifact.

A second group of inhibitors described in the literature has been classified under the heading of chalones or chalone-like substances. Chalones are defined as "tissue-specific, species-nonspecific products of differentiated cells that selectively inhibit early cells of the same lineage" (Bullough and Rytomaa '65; Cline and Golde, '79).

Rytomaa and Kiviniemi ('68a,b) were the first to describe a low molecular weight inhibitory substance in serum of normal and leukophoresed rats as well as in the extracts of peripheral blood and peritoneal exudate cells. The factor, presumably derived from mature granulocytes (although this was not proved), could apparently inhibit the uptake of tritiated thymidine in granulocytic cells. Similarly, an erythrocytic chalone derived from mature erythrocytes was found to have a specific inhibitory effect on cells of the erythrocyte lineage (Kivilaskso and Rytomaa, '71; Bateman, '74; Lord et al., '74a; Mariyama, '78).

A number of other chalone type substances have been described, some of which were shown to inhibit the growth of

in vitro granulocyte-macrophage colonies (Paran et al., '69; Paukovits, '71; Shadduck, '71; Lord et al., '74a,b). In one particular study, growth of bone marrow cells in an in vivo diffusion chamber was reduced following the injection of a granulocyte-derived inhibitor, indicating that such substances might be physiologically active (Mac Vittie and McCarthy, '74). Although a number of studies have claimed target cell specificity for these inhibitory substances, others have failed to do so (Herman et al., '78). Thus, as with the lipid-containing serum inhibitors, the significance of these chalone type regulators will not be fully understood until they are better characterized.

Another category of possible inhibitors of granulopoiesis and macrophage production are the neutrophil-derived factors that affect the GM-CSF producing cells. One group has reported that extracts of human and mouse neutrophils will inhibit GM-CSF production by monocytes and macrophages and thus inhibit endogenous colony formation (Broxmeyer et al., '77, Borxmeyer, '78). A more recent study suggests that this granulocyte-derived inhibitory factor is lactoferrin, the iron-binding protein present in the specific granules of mature granulocytes (Broxmeyer et al., '78).

Prostaglandins of the E series (PGE) are also candidates for negative regulators of hemopoiesis (Kurland and Moore, '76, '77; Kurland et al., '78). Macrophages,

which produce PGE, will inhibit G.M. colony formation if cultured at a high density as a feeder layer beneath the semisolid agar. Addition of indomethacin (an inhibitor of PGE) reduces the levels of PGE as determined by a radioimmunoassay and increases the number of hemopoietic colonies found in the cultures. The target cell for inhibition in this system appears to be the GM-CFC rather than the GM-CSF producing cell. Other studies showing that PGE inhibits the in vitro growth of a variety of tumor cell lines suggests that it might function on an inhibitor of all hemopoietic cells rather than just the granulocytic and macrophage lines of differentiation (Kurland and Moore, '76).

In addition to the negative regulators discussed thus far, a variety of other inhibitory substances have been described in the literature. For example, a factor has been found in normal mouse marrow that will inhibit the proliferation of CFU-S in regenerating spleens (Lord et al., '76). Since this negative factor can inhibit the induction of CFU-S proliferation initiated by the stimulatory factor found in regenerating marrow (see section d), it has been postulated that the proliferative status of the CFU-S is determined by the ratio of these two factors in the marrow. Along a different line, it has been shown that the serum of polycythemic mice contains a factor that will depress erythropoiesis in normal animals (Krymowski and Krymowska, '62; Whitcomb and Moore, '64). Finally, it has been reported

that interferon will inhibit granulocyte and macrophage colony formation in vitro (Fleming et al., '72; McNeill et al., '73). Interferon also inhibits the growth of a variety of other normal and neoplastic cells in vitro and thus the physiological significance of this substance as a specific inhibitor of granulopoiesis and macrophage production is questionable.

In conclusion, a number of the inhibitors discussed may represent negative regulators that function in vivo whereas others are likely artifacts of the various systems used in the studies. With the introduction of more purified cell populations and regulators, a large number of questions concerning negative regulation of hemopoiesis will be answered in the near future.

2) Cellular Regulation

a) T-Cell Regulation

It is now apparent that T-lymphocytes are involved in the regulation of hemopoiesis through their involvement in the production of long range regulators (MEG-CSF, EO-CSF and GM-CSF) (Ruscetti and Chervenick, '75; Metcalf and Johnson, '78). There is, however, evidence suggesting that T-lymphocytes might interact with hemopoietic cells at a more localized level, perhaps through the secretion of short range factors or by actual cell-cell contact.

The early observations of Auerbach ('63) and Metcalf

('64), that thymic tissue enhances the growth of spleen grafts both in vitro and in vivo, indicated that the thymus might be involved, to some extent, in hemopoietic differentiation. Other findings, such as induction of anemia in the mouse (Metcalf, '65) and arrest of erythroid maturation in the opposum (Miller et al., '65) following thymectomy as well as erythroid aplasia in humans suffering from thymomas (Fisher, '64; Roland, '64), further support the notion that the thymus plays some role in hemopoiesis.

More recent studies have shown that thymectomy leads to a decrease in (a) the number of cycling CFU-S (Frindel and Craizat, '73), (b) the number of marrow CFU-S, particularly those which give rise to granulocytic colonies (Trainin and Resnitzky, '69), and (c) the proliferative capacity of the bone marrow cells (Zipori and Trainin, '75). Furthermore, it has been shown that bone marrow cells from both thymectomized and nude mice are considerably less effective than normal marrow cells in their ability to protect against radiation death (Zipori and Trainin, '73, '75).

Using a different approach, Goodman and her colleagues found that the poor growth of parental bone marrow cells in F1, hybrid recipients in certain strain combinations could be augmented by the simultaneous injection of radiation sensitive thymocytes syngeneic with the parental grafts (Goodman ad Shinpock, '68). The augmentation of parental marrow growth did not appear to be simply a secondary effect

resulting from a weak graft-versus-host reaction, since thymocytes from mice tolerant to the F1 histocompatibility antigens were equally as effective (Goodman et al., '72). Lymph node cells (LNC), while not showing any enhancement of the parental graft as measured by ^{59}Fe uptake, did significantly increase the number of macroscopic and microscopic colonies found in the recipient spleen (Basford et al., '74). In addition to increasing the number of detectable spleen colonies, LNC injected with the marrow cells tended to shift the predominant colony type from erythrocytic to granulocytic. Although the function of the thymocytes or LNC in this system is not known their effect appears to be mediated by a short range factor or cell-cell contact since thymocytes implanted in a cell impermeable diffusion chamber lost their ability to augment the survival of the marrow graft (Goodman and Shinpock, '72).

More evidence in favour of T-cell regulation of hemopoiesis is provided by studies using congenitally anemic W/W^V mice. The anemia of these mice can be completely cured by the injection of normal stem cells (Bernstein and Russell, '59). It was recently discovered, however, that the ability of normal marrow to function in this manner is lost if it is treated with anti-theta serum prior to injection (Wiktor-Jedrzejczak et al., '77; Sharkis et al., '78b). Injection of normal, but not W/W^V thymocytes, along with the anti-theta treated marrow again resulted in increased hematocrit values in the W/W^V recipients. Anti-theta

treatment of the donor marrow also resulted in a shift from erythrocytic to granulocytic spleen colonies, an effect contrary to that observed by Basford et al. ('74). This apparent shift in differentiation within the recipient spleen could be reversed by the injection of normal thymocytes. From these studies, it was hypothesized that normal, but not W/W^{v}, marrow contains a theta-sensitive regulator cell (TSRC) that is in some way involved in the regulation of hemopoietic differentiation. Other studies showing that the effect of the thymocytes can be exerted from within a diffusion chamber or that it can be replaced by the injection of thymosin (Sharkis et al., '78a) suggest that direct cell contact with the bone marrow cells is not necessary.

The studies described in this section, along with those showing that T-lymphocytes are required for efficient human erythroid burst formation (Nathan et al., '78), strongly support the notion that T-cells play a major role in hemopoiesis and most likely exert their effects at several stages of differentiation.

b) Stromal Cell Regulation

Regulation of hemopoiesis by the non-hemopoietic "stromal" cells of the blood-forming organs is probably the least understood aspect of regulation. Since the demonstration that hemopoietic cells are derived from blood-

borne precursors, it was obvious that the blood-forming organs must contain a specialized class of cells, not found in other organs and capable of supporting hemopoietic differentiation.

Conclusive evidence indicating that the organ stroma is involved in hemopoiesis comes from studies on the anemic Sl/Sl^d mice. Although the stem cells of these mice function normally when transplated into irradiated recipients, their spleen and bone marrow appear to have a greatly decreased capacity to support hemopoiesis (McCulloch et al., '65; Berstein, '70; Altus et al., '71; Fried et al., '73; Wolf, '78). Thus, when bone marrow cells from normal (+/+) mice are injected into Sl/Sl^d recipients, very few colonies develop in the spleen. The observed effect is not the result of a suppressive factor since normal spleens, when transplanted into Sl/Sl^d mice, support hemopoiesis perfectly well and cure the anemia of the host (Bernstein, '70; Altus et al., '71; Trentin, '71; Fried, '73). Furthermore, the product of the Sl/Sl^d gene does not appear to be a long range humoral factor, since parabiosis of the anemic mice to normal mice did not enhance the hemopoietic capabilities of the Sl/Sl^d spleen or marrow (McCulloch et al., '65).

The role played by the stromal cells in normal hemopoiesis is at present a controversial issue.

Trentin and his colleagues believe that the stromal cells actually direct the differentiation pathway of the

CFU-S (Wolf and Trentin, '68; Trentin, '70, '71). They found that hemopoietic colonies develop in the bone marrow as well as in the spleen of irradiated reconstituted mice (Wolf and Trentin, '68). These bone marrow colonies are predominantly granulocytic (erythroid:granulocytic or E:G ratio of <1) while those in the spleen are mainly erythroid (E:G ratio of 3.5). The predominance of granulopoiesis in the marrow is not the result of a selective seeding of granulopoietic stem cells, since cells freshly seeded into the marrow will initiate normal numbers of erythropoietic colonies in the spleens of a secondary host (Trentin, '78). In experiments in which pieces of marrow were grafted directly into the spleen, it was found that individual colonies (clones) which spanned over the graft site contained granulocytic cells on the marrow side and erythrocytic cells on the spleen side. These findings strongly suggested that the stroma of the organ greatly influences the particular line of development within a colony. Upon closer analysis of colony development within the spleen, these investigators also noted that certain types of colonies will only develop in certain areas (Curry et al., '67b).

Attempts to reduce the number of erythroid colonies and increase the number of granulocytic colonies by hypertransfusion (suppression of endogenous EPO) have failed. Instead, one finds no change in the number of granulocytic colonies in such mice and only small aggregates of undifferentiated cells in place of the large erythrocytic

colonies (Curry et al., '67; Wolf and Trentin, '68). Following injection of EPO, these small aggregates quickly develop into erythroid colonies. The interpretation of these findings is that the small aggregates of undifferentiated cells are EPO sensitive cells derived from the CFU-S by some EPO independent mechanism. It is suggested that the stromal cells are involved in this differentiation step. From these observations it was postulated that the stroma of the hemopoietic organs is organized into spatially separate compartments, each one specifically inductive for a particular line of hemopoiesis. These individual inductive compartments became known as the hemopoietic inductive microenvironments (HIM) (Curry et al., '67; Wolf and Trentin, '68; Trentin, '70, '71, '78). Thus the spleen consists mainly of erythroid HIM while in the bone marrow granulocytic HIM predominates. Other studies indicate that the stromal cells responsible for supporting erythropoiesis (erythroid HIM) are morphologically distinguishable from those which support granulopoiesis (granulocytic HIM) (La Pushin and Trentin, '75).

A second hypothesis on the role of stromal cells in hemopoiesis proposes that these cells in the blood forming organs provide a proper environment for hemopoiesis, but are not specifically inductive (McCulloch et al., '73). This hypothesis was formulated from the observation that, with certain strains of mice, parental bone marrow grafts show a delayed growth in F1 hybrid recipients, a phenomenon known

as CFU repression or hybrid resistance (McCulloch and Till, '63; Cudkowicz and Stimpfling, '64). This resistance is not present until 3 weeks of age and it disappears approximately 7 days after irradiation (Cudkowicz, '68). The resistance does, however, return several months following repopulation with isologous cells, indicating that the phenomenon is mediated by a cell which turns over very slowly. McCulloch et al. ('73) have postulated that this cell is involved in hemopoietic regulation and that CFU repression is actually an example of abnormal hemopoiesis. These regulatory cells have become known as "managerial" cells and it has been suggested that they interact with the CFU-S through short range secreted factors. The major criticism of this hypothesis is that there is no evidence suggesting that the cells which mediate the CFU repression are also involved in the regulation of hemopoiesis.

Regardless of which line of thinking one adheres to, it is quite apparent that stromal cells play a major role in hemopoiesis. At this particular time, it is difficult to ascertain whether the effect is mediated via a short range factor or by cell to cell contact.

Knopse et al. ('66) have found that although the marrow stroma function of rats is relatively radiation resistant, permanent aplasia is found in these organs several months following irradiation. In experiments where part of the femur was shielded, the line of demarcation between the

hemopoietically active shielded portion and the irradiated (aplastic) area was found to be quite abrupt suggesting that the hemopoietic stromal elements themselves are not migratory and their influence is relatively localized. More recently, Wolf ('78) demonstrated that when +/+ and Sl/Sl^d spleens are grafted to each other, hemopoietic activity is confined to the +/+ side of the chimeric organ. In Sl/Sl^d spleens implanted with +/+ marrow or spleen stroma, hemopoietic activity is again restricted to the area of the graft. Very sharp lines divided the grafted hemopoietically active tissue from the hemopoietic tissue of the host spleen. These findings, consistent with those of Knopse et al., suggest that the effect of the stromal cells is not humoral (at least not long range), not easily diffusable and the cells themselves are not migratory.

Along another line, Mintz and Cronmiller ('78) have shown that tetraparental mice can be produced by injecting $SL^J/+$ tumor cells into a +/+ blastocyst. The organs of one of the mice derived from this procedure consisted primarily of $SL^J/+$ cells. Despite the apparently low number of normal cells, the animal appeared to be hematologically normal. The fact that presumably only a few (+/+) stromal cells were sufficient to overcome the defect in the anemic tumor cells suggests that the stromal effect is mediated by a short range factor rather than by actual cell contact. The major criticism of this interpretation is that the number of +/+ stromal cells in the spleen and bone marrow of this mouse

could not be determined.

Although most repopulation experiments suggest that the function of the hemopoietic organ stromal cells is radiation resistant, a number of recent studies indicate that irradiation does have an effect on these cells. As discussed previously, Knopse et al. ('66) found that marrow hemopoiesis is severely inhibited several months following irradiation of the femur. In a series of transplantation experiments, Fried et al. ('73) showed that irradiated (950R) spleen and marrow have a decreased ability to support hemopoiesis. More recently they found that irradiated femurs (1000R) contain considerably fewer CFU-S than non-irradiated controls (Fried et al., '76). These studies suggest that the stromal cells do turn over, although probably slower than the CFU-S. Evidence that stromal cell precursors can repopulate a hemopoietically inactive environment is provided by the studies of Knopse et al. ('68) who found that injection of normal marrow cells directly into the cavity of an irradiated femur results in the regeneration of a functional stroma. Thus, it appears as if the stroma of the hemopoietic organs can be regenerated from non migratory precursor cells.

Attempts at propogating the stromal cells in vitro have met with some success. Friedenstein et al. ('74, '78) were able to culture adherent, "fibroblast-like", spleen and bone marrow cells that initiate hemopoietic foci upon

transplantation beneath autologous and semi-syngeneic kidney capsules. The fact that the majority of the hemopoietic cells within the foci was derived from the host suggests that the donor cells provided a proper environment for hemopoiesis.

Using an improved culture system, Dexter et al. ('77) and Dexter and Moore ('77) found that adherent cells from +/+ but not Sl/Sl^d marrow would support the proliferation of CFU-S in vitro. More recently, it has been shown that these adherent cells will support the development of GM-CFCs, MEG-CFCs, and BFU-E, presumably from the CFU-S (Murphy et al., '78; Testa and Dexter, '78). Since no humoral regulators can be detected in these cultures, these findings are consistent with the interpretation that the very early stages of hemopoiesis (committment to a single line of differentiation), is controlled by EPO and CSF independent mechanisms; perhaps by the adherent stromal cells.

Despite the introduction of these culture systems, very little is known about the stromal cells involved in hemopoiesis. Until these cells can be characterized it will be very difficult to determine whether they are actually stimulatory, as the HIM model proposes, or whether they serve to support hemopoiesis, as the "managerial" cell model suggests.

G. Development of the Research Project

A review of the literature has indicated that cellular regulation is an important aspect of hemopoietic differentiation. In spite of this, very little information is available regarding the different cell populations involved in hemopoiesis, especially on the stromal cells of the hemopoietic organs. This lack of knowledge stems from the fact that most models have been designed to study the hemopoietic cells (CFU-S, GM-CFC, etc.) and not the regulatory cells.

B.M. Longenecker observed that inoculation of chick embryo spleen cells onto the chorioallantois membrane (CAM) of a host embryo results in the development of hemopoietic colonies. We felt that this system could be used as a model to study hemopoiesis (particularly the cell interaction aspect) for the following reasons: (1) It is generally accepted that hemopoietic cells require a proper "environment" for differentiation. That hemopoietic colonies developed on the CAM suggested that either a cell population in the donor inoculum or one in the CAM was providing this environment. Since both the donor cells and the host embryos could be manipulated and since the colonies were quite accessable throughout their development, we felt that the system offered an excellent opportunity for defining and characterizing the different cell populations involved. (2) It is an in vivo model; therefore we would not have to

deal with artifacts introduced in an in vitro system. (3) Since the embryo is accessable at all stages of development, the system is ideal for studying the ontogeny of the various cell populations present in the CAM colonies. (4) Finally, a large variety of chicken lines were available to us for genetic studies.

Chapter II

Materials and Methods

Eggs. Inbred SC eggs (B^2/B^2) were purchased from Hyline International, Johnston, Iowa. Fertile eggs from random bred chickens were supplied by the University of Alberta poultry research farm. Japanese quail eggs were obtained from Bioscience Animal Services at the University of Alberta.

Cell Suspension. Unless otherwise stated, spleens from 14-day chick embryos were used as a source of cells. In the initial experiments, cell suspensions were prepared by gently pressing the spleens through a wire sieve. Cell suspensions prepared in this manner contain single cells as well as cell aggregates, both of which were included in the inoculum unless otherwise stated. For characterization of the CAM colony-forming cells, spleens were teased apart with forceps and then treated with a collagenase-DNAse solution (.25% collagenase and .01% DNAse in Ca, Mg free Hank's balanced salt solution) for 60 minutes at 37°C. DNAse was added to prevent the formation of aggregates due to DNA polymerization in the medium. Throughout the dissociation procedure the suspension was vortexed every 15 minutes. Following the enzyme treatment, the few remaining small aggregates were dispersed by passing them through a 22 gauge syringe needle. The resulting suspension contained only single cells. Cell viability was determined by eosin dye exclusion.

Aggregation. Cells were reaggregated in vitro as described by Moscona ('61). Twenty-five ml Erylenmeyer flasks containing $20x10^6$ cells in 3 ml of medium were rotated on a gyrotory shaker at 70 rpm for 20 hours at 37°C. The medium consisted of Hepes buffered MEM (Gibco) without sodium bicarbonate but supplemented with 10% fetal calf serum (Gibco) and 10% chicken serum (Gibco).

Preparation of host embryos. In all experiments, 12 day embryos were used as hosts. In preparation for inoculation, the egg shell was punctured in two places (using a 22 guage stainless steel needle); at the tip of the air sac and on the uppermost side, midway along the long axis while the egg was held with the long axis horizontal. The second puncture was a small slit, carefully made to avoid touching the CAM. A gentle vacuum applied to the first puncture produced a second air sac directly beneath the second puncture. The egg was then rotated 90 degrees on its long axis, adjusting this new air sac under an undamaged part of the shell. At this point, the shell was burred from an area of 1.0x1.5 cm; the exposed shell membrane was softened with a drop of paraffin wax and then stripped away with blunt forceps. Cells or aggregates were inoculated in a 0.1 ml volume through the opening directly onto the exposed chorioallantoic membrane (CAM). Following inoculation, the hole in the shell was covered with masking tape and sealed with melted paraffin wax.

Harvesting the CAMs and scoring the colonies. At the time of harvest, the top half of the CAM was cut away from the remainder of the embryo, washed several times in warm tap water and then stored in saline at 4°C. The membrane can be stored for up to 1 week under these conditions.

Colonies on the membranes were scored with the aid of a dissecting microscope (WILD), usually at 10x magnification. The groups were always coded prior to counting.

Adherence. Cells were allowed to adhere to 100 mm plastic tissue culture dishes (Corning) for 2 hours at 37°C. For maximum efficiency, no more than $80x10^6$ cells suspended in 10 ml of MEM with 10% FCS were added to each plate. After 2 hours, nonadherent cells were removed by washing and transferred to new culture dishes for an additional 90 minute incubation. Cells which failed to adhere during the second incubation period were considered to be the nonadherent fraction. The adherent cells were washed with 10 ml of Hank's balanced salt solution and then harvested by scraping with a rubber policeman.

Velocity sedimentation. Cells were separated on the basis of size using a "Sta-put" apparatus (17.5 cm diameter) as described by Miller and Phillips ('69). A maximum of $4x10^8$ spleen cells was allowed to settle (at 1 g) through a 5-30% fetal calf serum gradient for 3.5 hours. The first 250 ml of liquid collected from the gradient contained no cells

and was discarded. The remaining portion of the gradient was collected in 15 ml fractions (approximately 40) and the total number of cells in each was determined with a Coulter Counter. The sedimentation velocity of each fraction was calculated using a computer program, as described by Armstrong and Kraft ('73).

Equilibrium density centrifugation. The method used for density separation was essentially that of Shortman ('68). After the trace amounts of contaminating salts were removed from the BSA powder (by dialysis), it was dissolved in a salt solution of the following composition: 0.168M NaCl, 121 vol.; 0.168M KCL, 4 vol.; 0.112 M $CaCl_2$, 3 vol.; 0.168M KH_2PO_4, 1 vol.; 0.168M $MgSO_4$, 1 vol. A continuous 6--->26% (w/w) BSA gradient was then generated in a 5 ml cellulose nitrate tube (Beckman).

The gradient mixer consisted of two identical vertical chambers [one for the dense solution (26% BSA) and the other for the light solution (6% BSA)] connected at the base by a small tube. The chamber with the dense solution contained a wire mixer as well as a valve for draining the gradient into the centrifuge tube. The light BSA solution enters the base of the chamber containing the dense solution, mixes with the dense solution, and this mixture then drains into the centrifuge tube. Cells (a maximum of $1.2x10^8$) were introduced with the light BSA solution so they would be distributed throughout the gradient. After the gradient was

prepared, the tube was spun in a SW 50.1 swing out head rotor (Beckman) at 3500 g for 45 minutes. Following centrifugation, a sharp, hollow needle was inserted through the bottom of the tube (and through the pellet) and 25 fractions containing 12 drops each were collected. The pellet, also considered a fraction, was recovered after the gradient was harvested. The refractive indices of individual fractions from a blank gradient were measured and these values were used to calculate the density of the BSA in each fraction using the relationship between refractive index, density and BSA concentration described by Leif and Vinograd ('64) and by Bishop and Prentice ('66).

In Vitro hemopoietic colonies. The culture system for growing chicken hemopoietic colonies in vitro was adopted from that used for growing mammalian cell colonies (Metcalf, '77). The cells were suspended in medium consisting of .3% agar (Difco) in MEM supplemented with 10% each of fetal calf serum and chicken serum. The medium was prepared by mixing equal volumes of double strength MEM with equal volumes of an appropriate agar solution. A typical culture consisted of the following: 40% double strength MEM, 40% agar solution (.75%), 10% chicken serum and 10% fetal calf serum.

Serum from a chicken injected 6 hours previously with endotoxin (50 mg LPS per bird) provided an excellent source of GM-CSF (Dodge and Hansell, '78). The serum containing the factor was inoculated onto each culture (.1 ml per dish)

after the agar had solidified. Cultures were incubated at 37°C in a 10% CO_2 atmosphere for 6 days. During the scoring, no distinction was made between clusters and colonies.

Cellular radioimmunoassay. Details of this assay have been published (Longenecker et al., '78). Briefly, the cells were treated with antispecies serum for 30 minutes (room temperature), washed 3x and then incubated with excess ^{125}I rabbit anti-chicken IgG for an additional 30 minutes. Following the second incubation, the cells were again washed 3x and the radioactivity in the cell pellet was determined with a gamma counter.

Preparation of antispecies sera. Chicken anti-quail and quail-anti-chicken sera were produced following three weekly immunizations with approximately 2 cc of whole blood at each immunization. The first two immunizations were administered intravenously and the third one was given intramuscularly. Ten days following the third immunization, the animals were bled and the serum was collected and stored at -70°C.

Histology. Colonies along with the adjacent portion of the CAM were fixed in 10% formalin, processed, cut at a thickness of 5 microns and stained with Hematoxylin and Eosin. For the cell marker experiments, Feulgen stain ('24) was used to distinguish quail cells from chicken cells (Le Douarin, '69, '73). For morphological identification of the cells, colonies were dissected free of the surrounding CAM and dispersed into a single cell suspension using the

collagenase treatment. The cells were then smeared onto slides and stained with Wright's stain or benzidine stain for hemoglobin.

Irradiation. Cells and embryos were irradiated with a Cs^{137} Gamma Cell 40 (Atomic Energy of Canada) at a dose rate of 100 rads/min.

Colony size. Colony size was determined using an optical grid in the eye-piece of a microscope at 15x magnification. Colonies from each membrane of each group were included in the size analysis.

Chapter III

Results

A. Cell Dose Response and Kinetics of CAM Colony Formation

Discrete white and pink colonies (Figure 1) developed on the chorioallantoic membrane (CAM) of the chick embryo following the inoculation of an embryonic spleen cell suspension containing both single cells and aggregates. These colonies did not reflect the classical graft versus host reaction (GVHR) to a major B histocompatibility antigen, as the number found in syngeneic donor-host combinations was the same as that found in allogeneic combinations (Table I). Further, in contrast to hemopoietic colonies, the GVHR pocks do not contain erythrocytes; most of them also have a characteristic centre of necrosis. The number of CAM colonies detected seven days post-inoculation was found to be directly related to the number of cells inoculated. On a log-log plot, the points between 10^5 and 10^7 cells were linear with a slope of 1.00, suggesting that a single limiting colony-forming unit initiated each colony (Figure 2). Results such as this would be expected if the unit was a single cell or a single aggregate of cells.

The following experiments were designed to distinguish between these two possibilities.

In the first experiment, colony formation was observed at different times after cell inoculation. Surprisingly, very small colonies could be detected on the CAM within 12

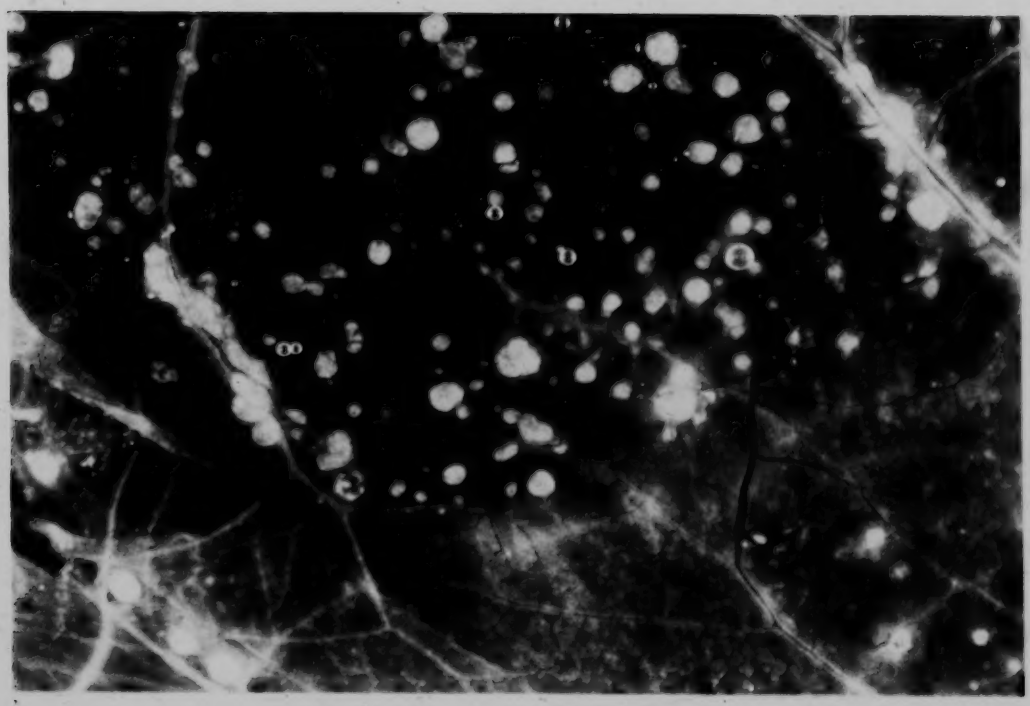

Figure 1. Hemopoietic colonies on the chorioallantoic membrane of a recipient embryo.

Table I Number of CAM colonies in syngeneic and allogeneic donor-host combinations.

Donor genotype(1)	Host genotype	Colony number (mean ± S.E.)
B^2/B^2	B^2/B^2	94 ± 17(2)
B^2/B^2	random bred	87 ± 13(2)

(1) $3x10^6$ donor spleen cells were inoculated on each CAM.

(2) Not significantly different.

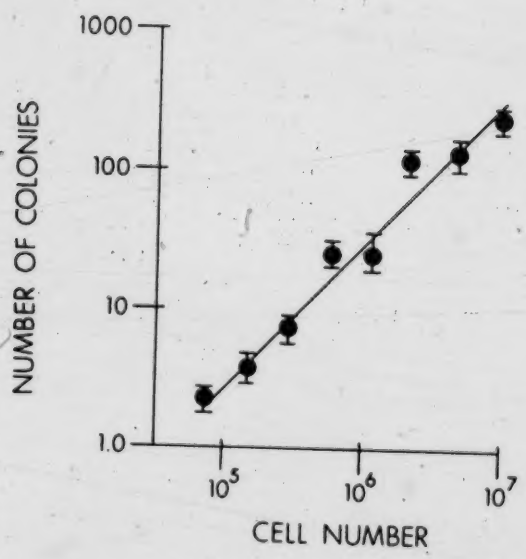

Figure 2. Relationship between the number of embryonic spleen cells inoculated and the number of hemopoietic colonies formed on the CAM. Each point represents the mean number of colonies obtained from twelve recipients. Vertical bars represent standard error of the mean.

hours of cell inoculation (Figure 3). The number of colonies increased slightly between 12 hours and 2 days and then remained constant throughout the observation period. The very early colonies were predominantly white in color; by five days post inoculation, however, some contained large numbers of mature erythrocytes giving them a "reddish" appearance. The presence of numerous CAM colonies twelve hours after inoculation suggests that they are derived from cell aggregates present in the inoculum. If this is true, a cell suspension should lose its colony forming capacity if the aggregates are removed prior to inoculation. To test this prediction, a spleen cell suspension was allowed to stand for 20 minutes and the resulting supernatant (containing single cells) and pellet (containing most of the aggregates) were inoculated onto host CAMs. A large number of colonies was found in the group inoculated with the aggregates (Table II). The single cells in the supernatant, on the other hand, initiated very few colonies. These findings strongly suggest that the aggregates found in the spleen cell suspension are responsible for CAM colony-formation.

B. Morphology of the CAM Colonies

For these studies, CAMs were harvested at various times following inoculation of embryonic spleen cell suspensions. The resulting colonies were either processed intact for histological sections or dissected free from the surrounding

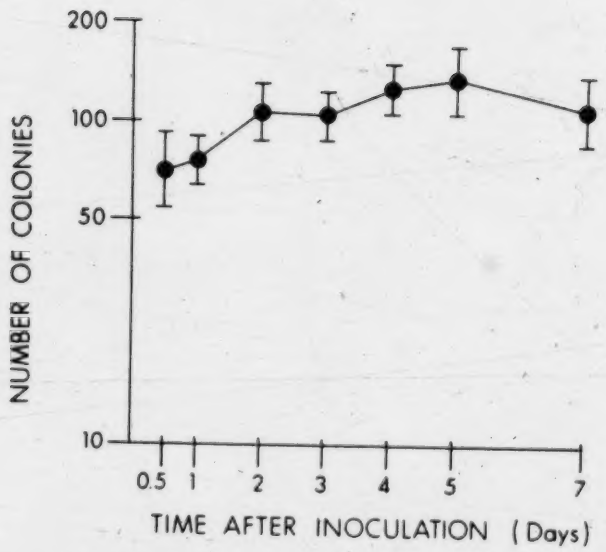

Figure 3. Number of CAM colonies found at various times following cell inoculation. Seven groups of 12 eggs each were inoculated at day 0, opened at different time periods thereafter and the colonies were counted.

Table II Colony formation by aggregates and single cells.

Inoculum[1]	Colony number (mean ± S.E.)
Supernatant	4 ± 1[2]
Pellet	59 ± 11[2]

(1) Aggregates were allowed to settle for 20 minutes and then both the supernatant and the pellet were inoculated. 1.0×10^6 cells were inoculated onto each CAM. The number of aggregates in each group was not determined prior to inoculation.

(2) Differ significantly ($p < .001$).

CAM and dissociated into single cell suspensions which were smeared onto slides and stained for detailed morphological studies.

Shortly after inoculation (12 hours), the young colonies appeared as aggregates on the epithelial surface of the CAM (Figure 4). These aggregates contained two morphologically distinguishable cell types: small intensely staining cells which resembled lymphocytes and larger, lighter staining cells with numerous cytoplasmic vacuoles (Figure 5). The cytoplasmic vacuoles do not contain fat as they failed to stain with Sudan IV. No mature hemopoietic cells could be detected in the aggregates at this time. By 24 hours post-inoculation, most of the immature colonies had penetrated the ectoderm and grown into the mesodermal layer of the CAM (Figure 6). A large number of colonies was already vascularized at this stage of development. In addition to the cells found in the inoculated aggregates, the 24-hour colonies contained a few mature erythrocytes and granulocytes as well as numerous undifferentiated cells with a high nucleus:cytoplasm ratio (Figure 7). The mature cells found in early stages of colony development most likely represent host cells present in the small segments of blood vessels dissected along with the colonies. Very few differentiating cells were detected in these early colonies.

By three days post-inoculation the majority of the colonies was found in the mesodermal layer of the CAM and

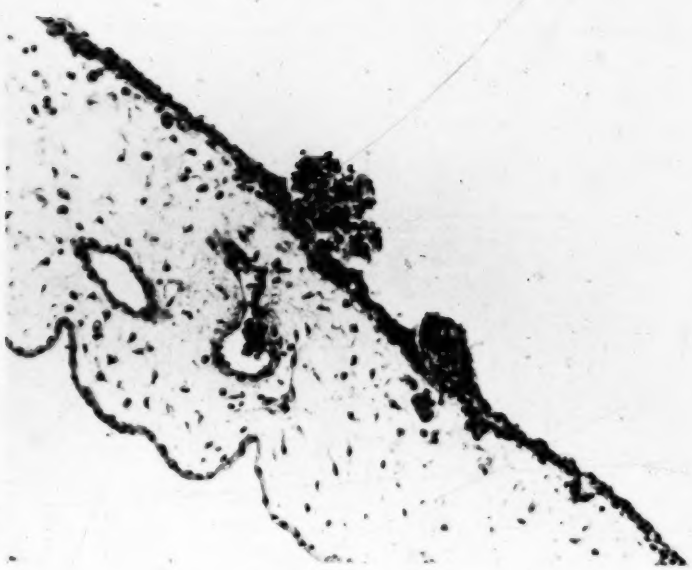

Figure 4. Two young colonies appear as cell aggregates on the outer (ectodermal) epithelium of the CAM, 12 hours post-inoculation (X190). Hematoxylin-Eosin stain.

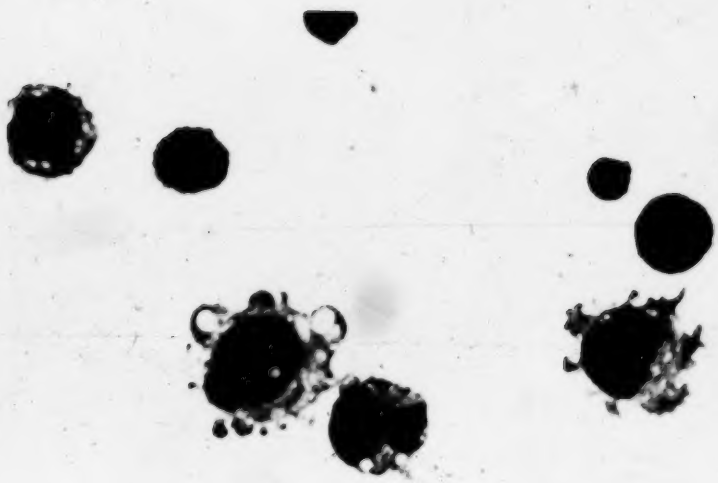

Figure 5. Small intensely staining cells and larger vacuolated cells found in the young aggregates (X1200) Wright's stain.

Figure 6. Hemopoietic colony has penetrated the ectoderm and partially grown into the mesoderm of the CAM, 1 day post inoculation (X190) Hematoxylin-Eosin.

Figure 7. Vacuolated cell and an undifferentiated cell with a high nucleus to cytoplasm ratio, both found in 1-day-old colonies.

most had increased in size considerably (Figure 8). Granulopoiesis was evident as large numbers of heterophil promyelocytes and mesomyelocytes could be detected (Figure 9). Differentiating cells of the basophilic and eosinophilic series were also present, although they appeared to make up a minor population of the granulopoietic cells. In contrast to the large number of cells in the granulocyte series, very few differentiating erythroid cells could be detected at this stage (Table III).

Seven days following inoculation, all stages of both erythropoiesis and granulopoiesis were present. In addition to promyelocytes and mesomyelocytes, these older colonies also contained a considerable number of metamyelocytes and mature heterophils (Figure 10). All stages of the eosinophilic and basophilic series could be detected, but again at very low numbers. In contrast to the younger colonies, erythropoiesis was evident in the 7-day colonies as significant numbers of immature benzidine positive cells were present in the smears (Table III, Figure 11). These findings strongly suggest that the colonies represent foci of hemopoiesis in the CAM.

C. Colony Formation by Single Cells vs. Reaggregated Cells

The experiments discussed previously indicated that aggregates of spleen cells give rise to CAM colonies. They did not, however, rule out the possibility that single cells

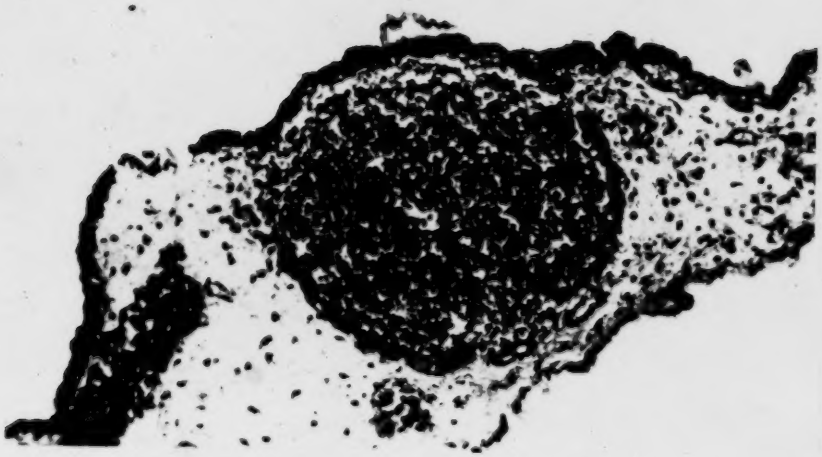

Figure 8. Three-day-old hemopoietic colony has fully penetrated the ectoderm and resides in the mesoderm of the CAM (X190) Hematoxylin-Eosin stain.

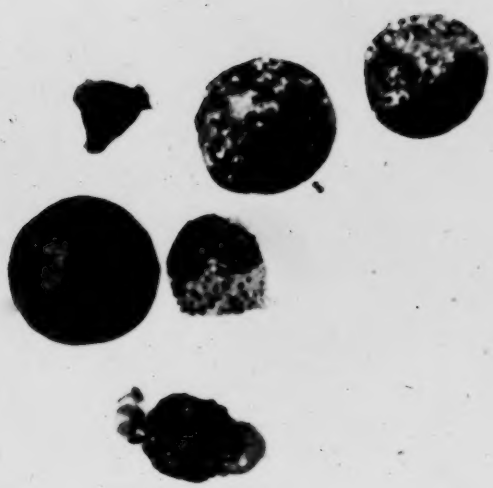

Figure 9. Cells from a three-day-old colony showing both the metagranuloblast and promyelocyte stages of granulopoiesis (X1200) Wright's stain.

Table III Differential erythroid cell counts.

Day after Inoculation	Number of immature cells[1] (% of total benzidine positive cells)
1	5 ± 3[2][4]
3	3 ± 2[3][4]
7	28 ± 1[2][3]

Each value represents the mean ± S.D. of three separate counts.

[1] Cells less mature than the late polychromatic stage were considered as immature.

[2] Differ significantly $p < .001$.

[3] Differ significnatly $p < .001$.

[4] Not significantly different.

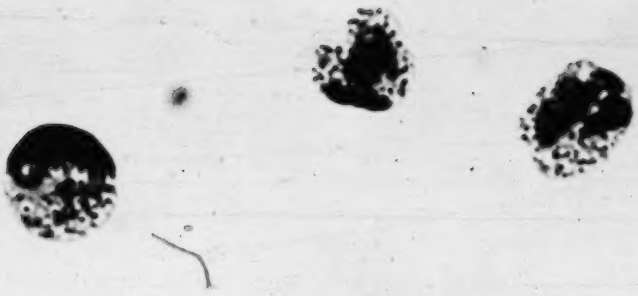

Figure 10. Seven-day-old colony cells with multilobulated nuclei characteristic of mature granulocytes (X1200) Wright's stain.

Figure 11. Three different maturation stages of benzidine positive cells found in 7-day-old pink colonies (X1200).

within the aggregates could also initiate such colonies. In the following experiment, embryonic spleens were completely dissociated by treatment with collagenase. The number of colonies formed by these cells was then compared to the number of colonies formed by cells that had been allowed to reaggregate prior to inoculation. One-half of the cell suspension was reaggregated in vitro as described in the Materials and Methods and the other half was cultured in large dishes to prevent any reaggregation. Twenty-four hours later, both groups of cells were harvested and inoculated at various dilutions onto host CAMs. Cell viability in each group was excellent (> 95%).

The reaggregated cell population contained numerous compact aggregates which initiated CAM hemopoietic colonies. The number of colonies formed was directly proportional to the number of aggregates present in the inoculum (Figure 12). Again, the log-log plot is linear with a slope that does not differ significantly from 1, indicating that each colony was initiated by a single unit. In contrast to the reaggregated cells, single cells formed significant numbers of colonies only in groups of embryos that had received more than 10^6 cells per host. The dependence of colony formation on a critical cell density suggests that the colonies resulted from cell aggregation on the CAM rather than from single cells. These findings strongly support the notion that only aggregates of embryonic spleen cells can initiate CAM hemopoietic colonies. One may argue that the greatly

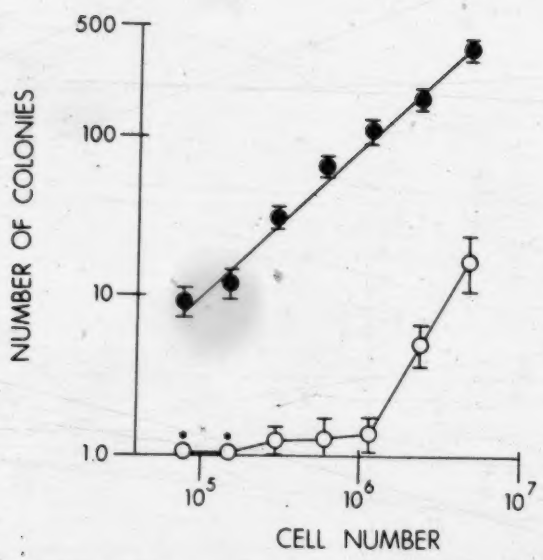

Figure 12. Colony-forming activity of single spleen cells compared to that of reaggregated spleen cells. Closed circles represent the mean number of colonies formed by cells which had been reaggregated prior to inoculation. Open circles are the mean number of colonies formed by non-aggregated cells. Vertical bars represent the standard error of the mean. The cell number at each dilution was based on the number of cells prior to culturing.
*No colonies were found on any of the CAMs in these groups.

reduced colony-forming ability of the single cells, compared to the reaggregated cells, reflects a loss of function of the cells when cultured in the non-aggregated form. Similar results were, however, obtained from experiments in which the non-aggregated cells were inoculated without culturing, clearly demonstrating that the above differences were not due to selective loss of function in the single cell cultures. Hereafter the aggregates will be referred to as CAM colony-forming aggregates (CAM-CFA) and the cells within the aggregates will be referred to as CAM colony-forming cells (CAM-CFC).

D. Origin of the Cells in the CAM Colonies

To determine whether the hemopoietic cells in the colonies were of host or donor origin, CAM colonies were initiated by inoculating CAM-CFA from chick embryo spleens onto the CAMs of Japanese quail embryos. Cells from these two species can be differentiated on the basis of a chromatin marker and by surface antigenic differences. The nuclei of Feulgen-stained quail cells show discrete chromatin clumps, in contrast to the more disperse pattern found in chicken cells (Le Douarin, '69, '73). Quail embryo CAMs were harvested at various times following inoculation of chick CAM-CFA. The resulting colonies were processed, sectioned and stained by the Feulgen method. Surprisingly, most one-day-old colonies already contained small numbers of host-derived cells. The number of these host cells increased

with colony maturation, so that by three days post-inoculation, numerous quail cells were found interspersed among the donor chicken cells (Figure 13). The appearance of quail cells in these young colonies suggests that immigrating host-derived cells might participate in the later stages of colony formation.

The extent of host contribution to the mature colonies was estimated using a cellular radioimmunoassay (CRIA) which differentiates between cells by antigens expressed on their surfaces. Longenecker et al. ('78) have recently shown that the CRIA can be used as a sensitive means of estimating the relative proportions of host and donor cells in chimeric cell population.

Five-day-old colonies initiated by chick CAM-CFA were dissected free of any surrounding CAM and dispersed into a single cell suspension. These cells were then used in the CRIA. Three groups of cells were used, originating from: (1) CAM colonies initiated by chick CAM-CFA onto chick CAM, (2) quail spleen, and (3) CAM colonies initiated by chick CAM-CFA onto quail CAM. Cells from each of the three groups were initially treated with either quail anti-chicken serum, chicken anti-quail serum, or were left untreated. To determine the amount of surface-bound antispecies serum, the cells were subsequently treated with ^{125}I rabbit anti-chicken serum which reacts with both chicken and quail IgG. The cells which were not pre-incubated with antispecies

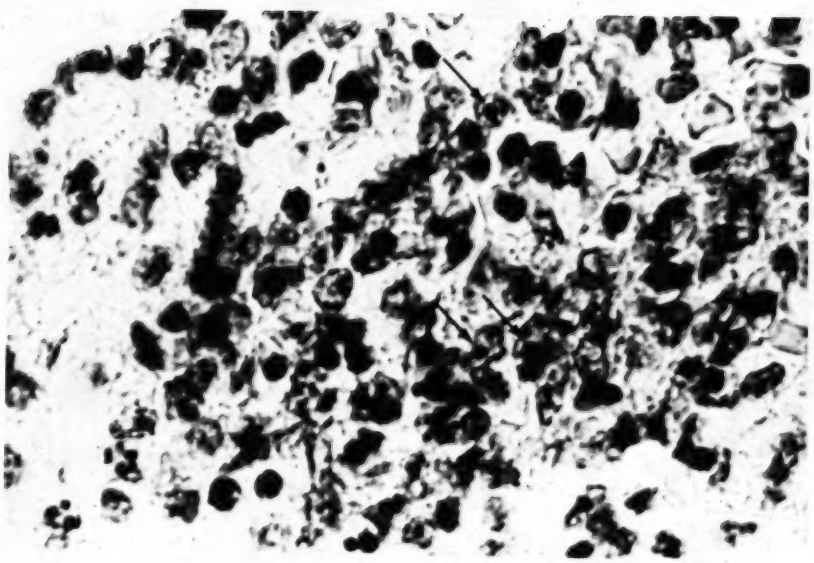

Figure 13. Section of a hemopoietic colony, derived from chicken CAM-CFA, inoculated onto a quail embryo CAM, 3 days post-inoculation. The clumped chromatin marker, characteristic of host quail cells (arrows), can be seen scattered throughout the colony (X480). Feulgen stain.

serum were also treated with the ^{125}I rabbit anti-chicken IgG as a control for intrinsic binding of the labelled antiglobulin. The results from the first two groups prove the specificity of the antispecies sera (Table IV). Results from the third group reveal that more than 75% of the cells in the mature colonies were host-derived.

The preponderance of host cells in the CAM colonies suggests that the donor cells do not contribute significantly to the hemopoietic content of the colonies and is consistent with the hypothesis that the donor cells belong to a population of non-hemopoietic cells which initiates hemopoiesis by host stem cells.

E. Cell Separation

If the donor CAM-CFC induce colony formation by host hemopoietic cells, it might be expected that the two cell types would have different physical characteristics.

Embryonic spleen cells, completely disassociated by collagenase treatment, were fractionated on the basis of adherence, size and density, and the individual fractions tested for CAM colony-forming ability and hemopoietic function. As no assay exists for the measurement of avian pluripotent stem cells, hemopoietic function was determined by the in vitro CFU-C assay, which detects committed hemopoietic progenitor cells (Metcalf, '77).

Table IV Origin of cells in CAM colonies as determined by the CRIA.

Origin of colonies		Origin of cells in the colonies	
Donor Cells	Host	Specific anti[2] quail counts (mean c.p.m. ± S.D.)	Specific anti[2] chick counts (mean c.p.m. ± S.D.)
Chick CAM-CFA	Chick	0	5235 ± 564[4]
Quail spleen[1]		5272 ± 580[3]	0
Chick CAM-CFA	Quail	4065 ± 1067[3]	1060 ± 13[4]

(1) Quail spleen cells (non inoculated) were used for the control as it was difficult to obtain sufficient numbers of quail CAM-CFC to initiate large numbers of CAM colonies.

(2) Both the control counts and the nonspecific counts were subtracted from these groups. Control counts represent the c.p.m. following treatment of cells with ^{125}I rabbit anti-chick sera in the absence of anti-species sera. Nonspecific counts represent the c.p.m. resulting from the nonspecific reactivity of the quail anti-chicken and chicken anti-quail sera.

(3) Not signicantly different.

(4) Differ significantly ($p < .005$).

1) Adherence

Chick embryo spleen cells were fractionated into adherent and nonadherent populations and tested for CFU-C and CAM-CFC. A small number of cells was used for the in vitro assay and the remaining cells were reaggregated and inoculated onto host CAM's. The adherent fraction, representing approximately 35% of the total spleen cell population, was primarily made up of cells that appeared to be somewhat smaller than the blast cells found in the different stages of hemopoiesis. These small, adherent cells formed very few in vitro colonies. Upon reaggregation and inoculation, however, they gave rise to large numbers of CAM colonies (Table V). The remaining 65% of the spleen cell population was nonadherent and consisted of large hemopoietic cells in all stages of differentiation. In contrast to the adherent population, the nonadherent fraction contained large numbers of CFU-C and very few detectable CAM-CFC. These experiments clearly demonstrate that the CAM-CFC are much more adherent than the hemopoietic progenitor cells of the embryonic spleen.

2) Velocity Sedimentation

Embryonic spleen cells were fractionated by means of sedimentation through an FCS gradient at 1g, a procedure which separates cells largely on the basis of size. The distribution profile was arbitrarily cut into five different

Table V Separation of CAM-CFC and CFU-C by adherence.

Fraction	Number of CAM[1] colonies/2.5x10⁶ cells (mean ± S.E.)	Total number of[2] colonies/fraction	Number of CFU-C/1.25x10⁴ cells (mean ± S.E.)	Total number[3] of CFU-C/fraction (X 10⁻⁵)
Adherent	82 ± 10	3969	14 ± 2	1.4
Nonadherent	<5	<5	326 ± 34	60.5
Unfractionated	17 ± 3		230 ± 19	

(1) Cells from each fraction were reaggregated and the resulting aggregates plus remaining single cells were inoculated onto CAM's at the equivalent of 2.5x10⁶ cells per host.

(2) Calculated from the frequency given in the first column.

(3) Calculated from the frequency given in the third column.

fractions (Figure 14). The majority of CFU-C was found in fractions I and II which contained the largest non-erythroid cells as well as mature erythrocytes (Table VI). Very few CAM-CFC were detected in these fractions. The slower sedimenting cells found in fraction III, IV and V gave rise to numerous CAM colonies, while forming only a few in vitro colonies. It is apparent from these experiments that progenitor cells belong to a population of cells which sediments more quickly than the CAM-CFC.

3) Equilibrium Density Centrifugation

Fractionation of embryonic spleen cells on a 6-26% BSA gradient at 3500 g resulted in a cell profile showing three major peaks (Figure 15). Five different fractions were tested for CAM-CFC and CFU-C; four from the gradient and one from the pellet. Most CFU-C were found in the densest fraction (I) of the gradient (Table VII). In contrast, CAM-CFC appeared to be spread across the first three fractions with most being found in fraction II. Very few CFU-C or CAM colony-forming cells were found in the lightest fraction (IV). Similarly the pellet, which represented the extremely dense leukocytes and mature erythrocytes, contained only a small number of CFU-C and no detectable CAM-CFC. These results demonstrate that hemopoietic CFU-C are considerably denser than most CAM-CFC.

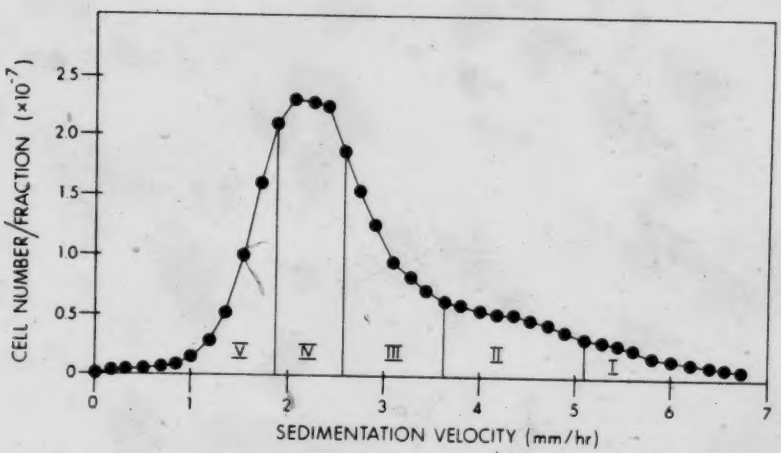

Figure 14. Sedimentation velocity profile of 14-day-old embryo spleen cells, following a 3.5 hour standing time. The total number of cells per fraction was determined using a Coulter counter.

Table VI Separation of CAM-CFC and CFU-C by velocity sedimentation.

Fraction	Number of CAM[1] colonies/2.5x10⁶ cells (mean ± S.E.)	Total number of[2] colonies/fraction	Number of CFU-C/1.25x10⁴ cells (mean ± S.E.)	Total number[3] of CFU-C/fraction (X 10⁻⁵)
I	<5	<5	664 ± 37	12.7
II	24 ± 9	390	661 ± 24	21.5
III	108 ± 8	2290	101 ± 4	4.3
IV	95 ± 7	2493	3 ± 1	0.2
V	47 ± 8	376	8 ± 3	0.1
Unfractionated	102 ± 8		434 ± 9	

(1) Cells from each fraction were reaggregated and the resulting aggregates plus remaining single cells were inoculated onto CAM's at the equivalent of 2.5x10⁶ cells per host.

(2) Calculated from the frequency given in the first column.

(3) Calculated from the frequency given in the third column.

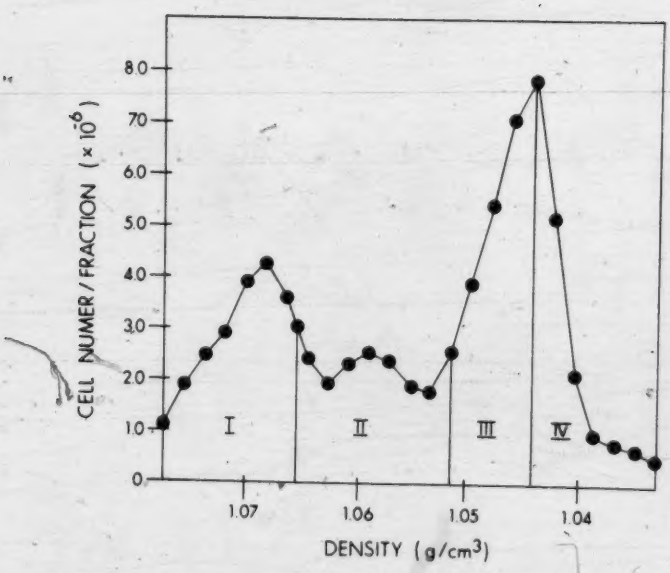

Figure 15. Equilibrium density centrifugation profile of 14-day-old chick embryo spleen cells, spun through a continuous 6-26% BSA gradient. The total number of cells per fraction was determined using a Coulter counter.

Table VII Separation of CAM-CFC and CFU-C by equilibrium density centrifugation.

Fraction	Number of CAM[1] colonies/2.5x10⁶ cells (mean ± S.E.)	Total number of[2] colonies/fraction	Number of CFU-C/1.25x10⁴ cells (mean ± S.E.)	Total number[3] of CFU-C/fraction (X 10⁻⁵)
I	53 ± 8	1414	881 ± 33	47.1
II	104 ± 10	1913	140 ± 6	5.2
III	67 ± 10	1399	25 ± 2	1.0
IV	35 ± 9	286	125 ± 6	2.0
Pellet	<5	<5	75 ± 5	1.2
Unfractionated	85 ± 10		393 ± 10	

(1) Cells from each fraction were reaggregated and the resulting aggregates plus remaining single cells were inoculated onto CAM's at the equivalent of 2.5x10⁶ cells per host.

(2) Calculated from the frequency given in the first column.

(3) Calculated from the frequency given in the third column.

F. Participation of Donor Hemopoietic Cells in CAM Colony Formation

The preceding experiments suggest that the CAM hemopoietic colonies result from an interaction between two distinct cell populations; donor-derived adherent spleen cells and host-derived hemopoietic cells. If this interpretation is correct, hemopoietic cells not derived from the host circulation should be able to participate in the formation of CAM colonies. In the following set of experiments, nonadherent spleen cells were mixed with CAM-CFA and inoculated onto irradiated host CAMs. We have previously shown that the nonadherent cell population of the spleen contains large numbers of hemopoietic cells (see above). Host embryos were given a total of 1400 rads of radiation (850 rads on day 5 and 550 on day 11) in an attempt to reduce the number of host hemopoietic cells capable of participating in colony formation. Results show that this amount of irradiation reduces (by 50%) the number of colonies resulting from the inoculation of CAM-CFA alone (Table VIII). The majority of colonies found on the irradiated CAMs were smaller and less well developed than those on the control membranes. In addition to these small colonies, some of the CAMs from the irradiated group contained extremely large red colonies, unlike any found in the nonirradiated control.

Inoculation of 1.5×10^7 nonadherent spleen cells mixed

Table VIII Participation of donor non-adherent spleen cells in CAM colony formation as determined by colony number.

Donor Inoculum	Host Irradiation[1]	Colony Number (mean ± S.E.)
CAM-CFA[2]	-	232 ± 17[3][5]
CAM-CFA	+	116 ± 10[3][4]
CAM-CFA + $1.5x10^7$ non-aderent cells	+	198 ± 20[4][5]
$1.5x10^7$ non-adherent cells	+	<10

[1] Host embryos were given a total of 1400R; 850 on day 5 of the incubation and 550 on day 11.

[2] The equivalent of $3.0x10^6$ adherent cells was inoculated onto each host embryo.

[3] Differ significantly ($p < .001$).

[4] Differ significantly ($p < .001$).

[5] Not significantly different.

with the aggregates onto irradiated hosts resulted in an increase in the number of colonies detected. Furthermore, colonies in this group appeared to be quite well developed, similar to those found on the control membranes (Table VIII). These observations suggest that the hemopoietic cells present in the nonadherent spleen cell fraction can participate in colony formation.

In the next set of experiments the CRIA was again used in an attempt to quantitate the contribution of the nonadherent spleen cells to CAM colony formation. Chick CAM-CFA and chick nonadherent spleen cells were inoculated onto quail CAMs. As before, the origin of the cells was determined with the CRIA.

In the first experiment, 10^7 nonadherent spleen cells plus $7x10^6$ adherent cells (CAM-CFC) were inoculated onto each host CAM. Colonies initiated by CAM-CFA alone contain very few donor-derived cells, as detected by means of the quail anti-chicken serum (Table IX, Exp. 1). Inoculation of nonadherent spleen cells together with CAM-CFA significantly increased the number of donor cells which could be detected in the colonies.

In a second experiment, $8x10^6$ nonadherent cells were inoculated together with the $7x10^6$ adherent CAM-CFC. One group of recipient embryos was irradiated prior to inoculation to try to reduce the number of host cells capable of participating in colony formation. In the non-

Table IX Experiment 1 - Participation of donor nonadherent spleen cells in CAM colony formation.

Origin of colonies		Origin of cells in the colonies		
Donor cells	Host	Control(2) (mean c.p.m. ± S.D.)	Total anti-chick counts (mean c.P.M. ± S.D.)	Specific(3) anti-chick counts
Chick CAM-CFA	Chick	1382 ± 216	9685 ± 116	7840
Quail spleen(1)		1189 ± 169	1652 ± 505	0
Chick CAM-CFA	Quail	1691 ± 225	2468 ± 417	314(4)
Chick CAM-CFA + 10^7 chick nonadherent spleen cells	Quail	1738 ± 622	3871 ± 544	1670(4)

(1) Quail spleen cells (not inoculated) were used for the control as it was difficult to obtain sufficient numbers of quail CAM-CFC to initiate large numbers of CAM colonies.

(2) Control groups represent c.p.m. following treatment of cells with ^{125}I rabbit anti-chick sera in the absence of antispecies sera.

(3) Both the control counts and the nonspecific counts, that is the counts resulting from the nonspecific reactivity of the quail anti-chicken and chicken anti-quail sera, were subtracted from these groups.

(4) Differ significantly ($p < .0125$).

irradiated groups, a large majority of the cells from the colonies initiated by CAM-CFA alone was of host origin (Table IX, Exp. 2). Addition of $8x10^6$ nonadherent spleen cells slightly increased the number of donor-derived cells detected in the colonies. The increase was not as dramatic as in the previous experiment, suggesting that a critical number of nonadherent spleen cells must be present before they can be detected in the CAM colonies. In contrast, colonies from the CAMs of irradiated embryos contained significantly more donor cells than colonies initiated by CAM-CFA in the absence of nonadherent cells. Once again, the results suggest that nonadherent embryonic spleen cells inoculated together with the colony-forming aggregates can contribute to the hemopoietic content of the CAM colonies. This contribution appears to be greatest when the number of host cells capable of participating in colony formation has been reduced by irradiation.

G. Irradiation of the CAM-CFA

Previous reports suggest that the murine hemopoietic organ stroma is relatively radioresistant (Jenkins et al., '70; Fried et al., '76). To determine whether CAM colony formation is affected by irradiation, CAM-CFA were exposed to a range of doses of gamma radiation prior to inoculation. Six days later, the number, size and cellular content of the resulting colonies was determined. Doses of radiation up to 1500 rads had little effect on the number of colonies formed

Table VII Experiment 2 Participation of donor nonadherent spleen cells in CAM colony formation

Origin of Colonies		Origin of Cells in the Colonies				
Donor cells	Host	Control[c] (mean c.p.m.±S.D.)	Total anti-quail counts (mean c.p.m.±S.D.)	Specific[d] anti-quail counts	Total anti-chick counts (mean c.p.m.±S.D.)	Specific[d] anti-chick counts
chick CAM-CIA	chick	3126 ± 422	8570 ± 698	0	18,080 ± 215	14,366
quail spleen[a]		3114 ± 605	20,541 ± 2384	11,983	3702 ± 616	0
chick CAM-CIA	quail	3021 ± 938	25,471 ± 3749	17,006	3687 ± 951	78
chick CAM-CIA + chick nonadherent spleen cells	quail	2997 ± 590	24,957 ± 6767	16,516	4488 ± 610	903[e]
chick CAM-CIA + chick nonadherent spleen cells	irradiated[b] quail	2358 ± 522	24,986 ± 4918	17,185	7531 ± 1958	4405[f]

(a) Quail spleen cells (non inoculated) were used for the control, as it was difficult to obtain sufficient numbers of quail CAM-CIC to initiate large number of CAM colonies.

(b) Host embryos in this group received 600 rads prior to inoculation.

(c) Control groups represent c.p.m. following treatment of cells with ^{125}I rabbit anti-chick sera in the absence of antispecies sera.

(d) Both control counts and nonspecific counts, that is the counts resulting from the nonspecific reactivity of the quail anti-chicken and chicken anti-quail sera, were subtracted from these groups.

(e) Counts in this group are not significantly different from the counts in the group in which only chick CAM-CIA were inoculated onto quail hosts (compare 903 to 78).

(f) Counts in this group are significantly greater than the counts in the group in which only chick CAM-CIA were inoculated onto quail hosts (compare 4405 to 78; $p < .025$).

(Figure 16). CAM-CFA which received 2500 rads, however, produced significantly fewer detectable colonies than the unirradiated controls. Closer analysis showed that, although the colony number was not greatly affected, the size of the resulting colonies decreased considerably following irradiation of the CAM-CFA, even with doses as low as 400 rads. The mean colony size decreased from a non-irradiated control value of 313 ±6 microns to a minimum of 164 ±4 microns following irradiation of the CAM-CFA with 1500 rads (Figure 17). The colonies initiated by the heavily irradiated aggregates were predominantly granulopoietic, suggesting that this function of the CAM-CFC is relatively radioresistant (Figure 18).

H. CAM Colonies Initiated by Spleens from Donors of Different Ages

In the following experiment spleens from embryos of different ages as well as from newly hatched chicks were tested for colony-forming ability. Cell suspensions were prepared by passing the spleens through nylon sieves. As before, both single cells and aggregates were inoculated.

All the embryonic spleens tested (11 day embryo to hatching) initiated hemopoietic colonies. Although the size of colonies initiated by the younger spleens was generally larger than that of the colonies from older spleen, the cellular content of most colonies was similar.

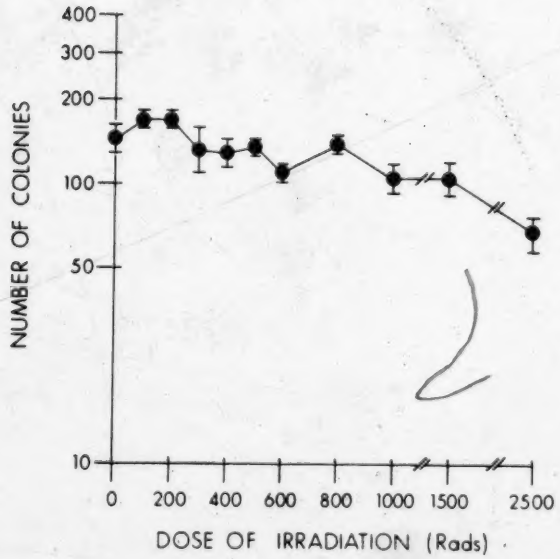

Figure 16. Effect of irradiating CAM-CFA with various doses of radiation on their ability to initiate CAM colonies. Vertical bars represent standard error of the mean.

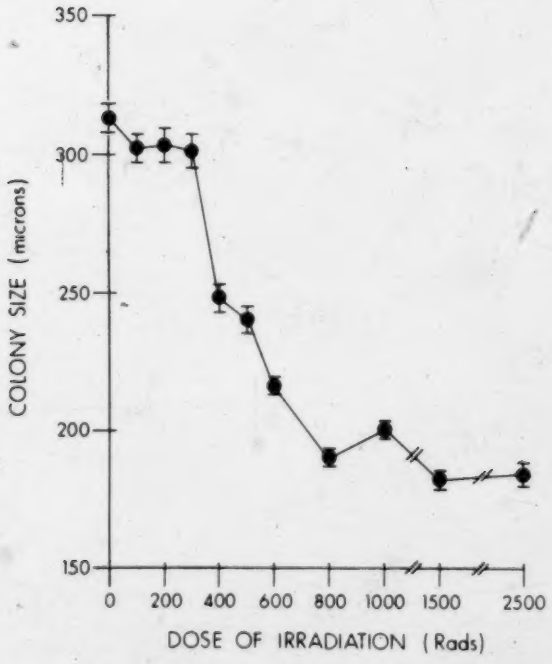

Figure 17. Effect of irradiation of the CAM-CFA on the size of the resulting CAM colonies. Each point represents the mean size of at least 300 colonies. Vertical bars represent standard error of the mean.

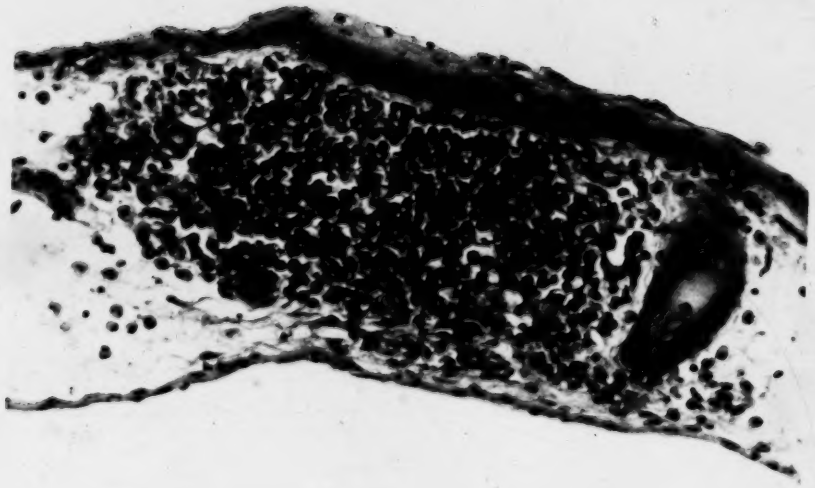

Figure 18. Section of a CAM colony initiated by a CAM-CFA irradiated with 1000R prior to inoculation.

On a per spleen basis, the largest number of hemopoietic colonies was obtained from spleens of embryos between the ages of 15 and 17 days incubation (Figure 19). After this stage of development, the number of colonies initiated by the spleen decreased as the age of the embryo increased. Very few colonies were derived from the inoculation of spleens from embryos at the time of hatching.

Spleen cells from two-day-old chicks, on the other hand, initiated primarily small white colonies that were quite different from those derived from embryonic spleens. Histological studies revealed that these small white colonies contain large numbers of cells with the morphology of small lymphocytes.

These "lymphoid" colonies are not graft-versus-host pocks to a major histocompatibility antigen since they are found when the donor and host carry the same "B" antigen. Furthermore, they probably are not the result of a graft-versus-host reaction to minor alloantigens, as these pocks are not found until 5 days post inoculation whereas the lymphoid colonies can be detected within 3 days of inoculation (Longenecker et al., '73). Whether the lymphocyte-like cells are derived from the host embryo remains to be determined.

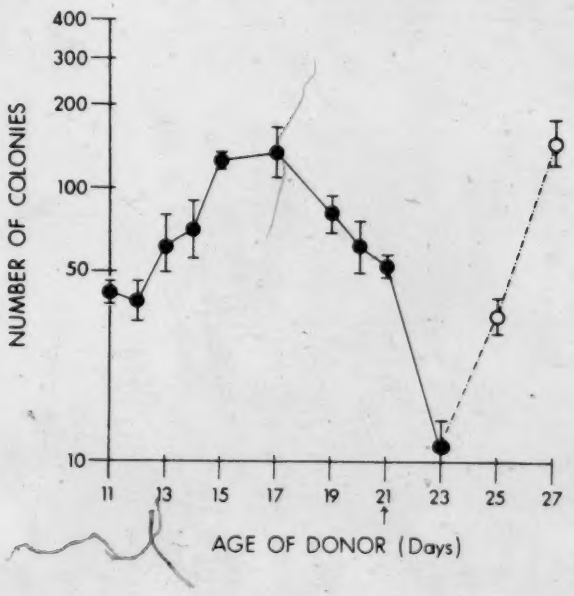

Figure 19. Ontogeny of the colony forming aggregates in the chicken spleen. Closed circles represent the mean number of hemopoietic colony forming aggregates per embryonic spleen at different days of development. Open circles represent the mean number of lymphoid colony forming aggregates per spleen from newly hatched chick. Donor and recipients were both B^2/B^2. Arrow along the abscissa indicates the day of hatching. Vertical bars represent standard error of the mean.

Chapter IV

Discussion

Although no assay exists for directly measuring the stromal cell function of the blood-forming organs, both in vivo repopulation studies (Curry and Trentin, '67; Curry et al., '67; Wolf and Trentin, '68; Trentin, '70, '71) and more recent long term in vitro experiments (Dexter et al., '77; Dexter and Moore, '77) have provided some information on the cell type(s) involved.

The most obvious characteristic of these stromal cells is that they provide a unique environment, suitable for hemopoietic differentiation. Whether the environment is specifically inductive remains a controversial issue. Compared to the CFU-S, the function of the microenvironment* cells is relatively radiation resistant, although it is affected by moderate amounts of irradiation (Fried et al., '76). This observation indicates that the function of the cells providing the proper environment in the hemopoietic organs is not dependent on proliferation. Finally, recent in vitro studies suggest that the stromal cells of the hemopoietic organs belong to an adherent cell population (Friedenstein et al., '74; Dexter et al., '77).

We have provided evidence, indicating that the CAM-CFC

*Microenvironment and stroma will be used interchangeably to describe the population of cells that support hemopoiesis in the blood-forming organs.

possess these characteristics and we therefore suggest that they represent the hemopoietic microenvironment or organ stroma of the chick embryo spleen.

The CAM-CFC, when aggregated and placed on the CAM of host embryos, initiate foci of hemopoiesis. These hemopoietic foci or colonies are not to be confused with the graft-versus-host pocks resulting from the inoculation of immunocompetent allogeneic lymphocytes. First, the number of colonies was the same regardless of whether donor and host embryos carried the same or different alleles of the chicken major histocompatibility complex. Second, the colonies contain cells at all stages of granulocytic and erythrocytic differentiation whereas pocks consist of mature granulocytes usually surrounding a characteristic necrotic center (Longenecker et al., '70).

Pocks and colonies have apparently been confused in a recent report by Jankovic et al. ('75). In a series of experiments that lacked genetic controls, they describe the development of lymphocytic, granulocytic, erythrocytic, thrombocytic and mixed "pocks" on the CAM following the inoculation of cells from different embryonic hemopoietic organs. The granulocytic, erythrocytic and mixed "pocks" most likely represented hemopoietic colonies that were induced by CAM-CFA present in the inoculum. Although the authors claim that thymus, bursa, bone marrow, peripheral blood and spleen will initiate these different pocks, in our

studies we found no pock or colony-forming activity in any embryonic organs other than the spleen (which only gives rise to colonies). The reason for this discrepancy is unknown.

Our cell marker studies have shown that the majority of cells within the hemopoietic colonies are of host origin, suggesting that the CAM-CFC are capable of supporting the differentiation of host-hemopoietic cells. That the CAM-CFA become vascularized shortly after inoculation and populated by host cells is in agreement with the studies of other investigators who observed similar events following the transplantation of pieces of whole spleen and thymus to the CAM (Moore and Owen, '67a; Moore and Metcalf, '71). In our studies the reaggregation step prior to inoculation most likely represents a reconstruction of small, functional aggregates from individual cells.

The undifferentiated cells with the high nucleus to cytoplasm ratio found in the 1 day old colonies appear to be similar to those described by Van Bekkum et al., ('71) as representing hemopoietic stem cells. Others have shown that pieces of thymus grafted to the CAM are initially populated by undifferentiated basophilic cells (Moore and Owen, '67a). They suggest that these cells represent an influx of host hemopoietic stem cells. It is possible that some of the undifferentiated cells found in the CAM-CFA shortly after inoculation are stem cells from the host's circulation,

since it has been shown by means of parabiosis experiments that the peripheral blood of the chick embryo contains cells capable of establishing life-long blood cell chimerism in the opposite partner (Havele et al., '79). Our results from the cell marker experiments are consistant with those of Friedenstein et al. ('74, '78) who found that hemopoietic foci initiated under the kidney capsule by fibroblast-like bone marrow or spleen cells are composed primarily of host derived hemopoietic cells. Nevertheless, the observation that the CAM-CFC can attract host hemopoietic cells does not prove that they are stromal cells.

Through a series of cell separation procedures, however, we were able to show that the CAM-CFC belong to a population of cells which is more adherent, less dense, and more slowly sedimenting than the hemopoietic progenitor cells of the spleen. Furthermore, in a series of preliminary experiments we found that adherent spleen cells were not able to initiate red blood cell chimerism when injected into host embryos of a different genotype. Chimerism was detected in the birds injected as embryos with nonadherent spleen cells. These findings strongly suggest that the CAM-CFC belong to a non-hemopoietic cell population of the spleen. The adherent characteristic of the CAM-CFC is consistent with the findings of Dexter et al. ('77) and Friedenstein et al. ('74) who have provided evidences suggesting that the cells which make up the hemopoietic environment of the blood forming organs of the mouse belong to an adherent cell

population.

As might be expected and in agreement with general concepts regarding hemopoietic organ stroma function, the ability of the CAM-CFC to initiate CAM colonies appears to be relatively radiation resistant, at least for the 7 day observation period. Although proliferation of the adherent cells is not a prerequisite for colony formation, the size analysis data suggests that under normal conditions, these cells do divide upon entering the CAM. Proliferation of the CAM-CFC would be expected to lead to an increase in the size of the unit capable of supporting hemopoiesis and hence an increase in the size of the colony. We were surprised to find a preferential loss of erythroid colonies following irradiation of CAM-CFA, an observation which suggests that the overall capacity of the CAM-CFC population to support erythropoiesis is more radiosensitive than its capacity to support granulopoiesis. This observation is similar to results obtained with mice, showing a selective loss of erythropoiesis in the spleen following large doses of radiation (Rauchwerger et al., '73a; Haley et al., '75).

Whether the CAM-CFC population contains a single cell type capable of supporting both erythropoiesis and granulopoiesis (managerial cell model) or whether it consists of several different cell types, each one specific for a particular line of hemopoiesis (HIM model) remains to be determined.

Most of the colonies do contain both erythropoiesis and granulopoiesis, though quite often one line of hemopoiesis predominates over the other. If the HIM model is correct, the fact that the majority of the colonies is mixed would imply that most CAM-CFA contain more than one type of CAM-CFC. That the induction of erythroid colonies appears to be more radiation sensitive than the induction of granulocytic colonies would be best explained by this model. If, on the other hand, the managerial cell model is correct, each CAM-CFA would be able to support both erythropoiesis and granulopoiesis. At the present time, our results can be interpreted to fit either model.

Although we have presented evidence suggesting that the CAM-CFC are stromal cells, the number of different cell types required for a functional CAM-CFA is not known. On a morphological basis, it appears as if the aggregates contain at least two distinct cell types; the small dense staining cells and the larger vacuolated cells. These cells may, however, simply represent different stages of maturation of the same population. If this is true, it is theoretically possible that a single cell could initiate a microcolony or perhaps support hemopoiesis in the absence of colony formation.

The adherent cell layer in the Dexter-type culture consists of three distinct cell types: phagocytic mononuclear cells with numerous cytoplasmic extensions,

flatened cells which tend to form a confluent monolayer and large (50-150 microns) lipid containing cells (Dexter et al., '77). These lipid containing or "giant fat" cells appear to be the most important, since in their absence, there is no CFU-S maintenance. Results from the Sudan IV staining procedure showed that the CAM-CFC contain no lipid material. The fact that the CAM-CFC are vacuolated is compatible with the suggestion that they may be phagocytic cells. It is interesting to note that adherent phagocytic cells are involved in the production of the factors (found in poke weed mitogen spleen cell conditioned medium) that initiate the mixed hemopoietic colonies in vitro (Metcalf and Johnson, '78). Although it is thought that these adherent cells are macrophages, they might in fact belong to the stromal cell population of the spleen. Whether the adherent cells actually produce the factor remains to be determined.

At this point, little can be said about the function of the CAM-CFC. That undifferentiated cells accumulate within the aggregates is consistant with the notion that the adherent cells secrete a chemotactic factor. This concept is in agreement with the studies of Le Douarin ('78) who has provided evidence suggesting that presumptive thymic tissue secretes a factor that attracts undifferentiated basophilic cells. This attractive stimulus diminishes after the thymus has received an influx of these cells. If differentiation of the hemopoietic cells within the aggregates requires a

stimulus from the CAM-CFC, it is most likely mediated via a short range factor or by direct cell contact, since hemopoiesis is found only within the defined area of the CAM-CFA and not in the surrounding CAM.

An interesting observation and one that deserves further investigation is that of the "lymphoid" colonies initiated by chick spleen cell aggregates. If the cells are lymphocytes derived from the host embryo, this finding would suggest that the CAM-CFC change at hatching from a cell population that supports hemopoiesis to one that supports lymphopoiesis. A change in the stromal cell function might, in part, be responsible for the change in the spleen at the time of hatching from a hemopoietic to a lymphoid organ. On the other hand, the lymphocyte-like cells might simply represent donor cells trapped in the colony forming aggregate. A dissociation and reaggregation experiment with spleens from newly hatched chicks would quickly resolve this problem.

The observation that the number of CAM-CFA per spleen increases and then decreases throughout the later half of fetal development is difficult to interpret. The initial rise in the number of CAM-CFA per spleen (from day 11-14) is most likely due to an increase in the size of the organ during this stage of development. The subsequent fall in the number of functional aggregates is somewhat puzzling. That this decline in activity occurs at the same time the spleen

normally begins losing its granulocytes suggests that the stromal cells at this stage have temporarily lost their attractiveness for hemopoietic cells and therefore are unable to initiate colonies. Alternatively, this fall in colony forming activity might be an artifact resulting from an increased sensitivity of the cells to the harsh experimental manipulations. Whatever the reason for this change in the potential colony forming activity, the observation certainly bears further investigation.

In conclusion, we suggest that the CAM assay represents the first binomial assay for the functional units which make up the hemopoietic organ stroma. It is the first system in which the cellular components of the hemopoietic microenvironment can be extensively manipulated without the loss of function. This has allowed us to partially purify these cells and characterize them with respect to adherence, size and density. Finally, we suggest that the CAM assay provides a new system for studying the regulatory function of the cells which make up the hemopoietic microenvironment and serves as a model for the development of similar assays in other species.

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Appendix I

Future experiments using the CAM assay:

1) The CAM-CFC can be further characterized (with a combination of cell separation techniques) to determine if more than one cell type is needed for a functional CAM-CFA.
2) If only one cell type is required, it should be possible to clone these cells using a protocol similar to that used by Friedenstein ('74). If the HIM model is correct, then perhpas some of the clones will be specifically inductive for a single line of hemopoiesis.
3) Using the millipore chamber technique or by establishing a culture system that promotes the survival of CAM-CFC, it might be possible to determine whether these cells secrete a factor that initiates hemopoiesis.
4) The ontogeny of the CAM-CFC (from 8 day incubation to 1 week post hatching) should be reinvestigated using the dissociation and reaggregation technique. This, no doubt, would provide some information on the origin of the lymphocyte in the "lymphoid" colonies.
5) Using the collagenase procedure, the other embryonic organs can be tested for CAM colony-forming activity.
6) Mouse spleen and perhaps human bone marrow could be used as a source of CAM-CFC.
7) The system is ideal for studying the effects of various drugs on the ability of the CAM-CFC to initiate colonies.